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continued by
C. HEYMANS
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A. F. DE SCHAEPDRYVER

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Pharmacokinetics of Anthracene-9-carboxylic Acid, a Potent Myotonia-Inducer

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Abstract—The pharmacokinetics of anthracene-9-carboxylic acid (9-AC), a potent myotonia-inducer, was determined in 9 New Zealand White male rabbits after the intravenous injection of 36 $\mu M \cdot kg^{-1}$. Myotonic contractions appeared 30 to 80 sec after the beginning of injection. One minute after injection, 25 % of the administered dose remains in the blood (9-AC in plasma + 9-AC in cells). Of the 9-AC present in whole blood, 78 % is in plasma and can be totally extracted with chloroform whereas the remaining 22 % is in the blood cells and can not be extracted. The disappearance of 9-AC from plasma can be explained numerically by assuming an open two-compartment model. By means of a non-linear regression analysis the equation $C_p = 20.65e^{-0.3216t} + 28.35e^{-0.0147t}$ was obtained. This equation gives the best fir for the mean values of all the experiments. The equilibrium between the two compartments is reached in 9.0 min and the $Vd_{ss} \cdot kg^{-1}$ at this time is equal to $273 \pm 18.6\ ml \cdot kg^{-1}$ which is only marginally larger (10 %) than the estimated extracellular volume ($246.0\ ml \cdot kg^{-1}$), suggesting that 9-AC barely penetrates into the cells. The plasma concentration values at $t_{1/2el}$ and $3t_{1/2el}$ are 1.07×10^{-4} M and 2.69×10^{-5} M, respectively. Similar concentration values were found by other authors as capable of reducing the chloride conductance of skeletal muscle membrane and producing myotonia *in vivo* and *in vitro*. Thus, there is a good correspondence between the concentrations active *in vitro* and the concentrations of plasma *in vivo* found in this study. Other pharmacokinetic parameters are: $t_{1/2\alpha} = 2.8 \pm 0.3$ min; $t_{1/2\beta} = 45.7 \pm 2.2$ min; Area under the curve (AUC) $= 1920 \pm 125.0\ \mu g \cdot min \cdot ml^{-1}$; $K_{12} = 0.085 \pm 0.01\ min^{-1}$;

$K_{21} = 0.162 \pm 0.02\ min^{-1}$; $t_{1/2el} = 28.84 \pm 2.2$ min., and $V_1 \cdot kg^{-1} = 172.1 \pm 9.7$ ml. All our findings plus the rapid appearance of myotonic signs and the correspondence with the *in vitro* studies support the previous assertion that 9-AC acts on the cellular membrane of the skeletal muscles.

Introduction

In recent years considerable attention has been given to chemically induced myotonias in mammalian skeletal muscle both *in vivo* and *in vitro* (Kwieciński, 1981; Laskowski and Dettbarn, 1977). Two main groups of chemicals have been studied: aromatic monocarboxylic acids and inhibitors of cholesterol synthesis. The basic mechanism of action of both groups of substances is the blockade of sarcolemmal chloride conductance (Bryant and Morales-Aguilera, 1971; Rüdel and Senges, 1973) although they differ in several other important aspects. For instance, the inhibitors of cholesterol synthesis cause profound morphological changes, not only in skeletal muscles (Somers and Winer, 1966) but also in other tissues and organs (cataracts (Peter *et al.*, 1973), testicular atrophy (Peter *et al.*, 1975)) whereas aromatic monocarboxylic acids apparently do not produce detectable structural changes even when they are administered daily up to 90 days (Ramos-Ramirez *et al.*, 1983). Some authors (Fairhurst *et al.*, 1976) think that the myotonia induced by hypocholesterolemic agents resembles *Myotonia distrophica.* On the other hand, Bryant and Morales-Aguilera (1971) emphasized that the changes induced by aromatic monocarboxylic acids are similar to *Myotonia congenita.* Numerous studies with the hypocholesterolemic agents *in vivo* have been performed (Winer *et al.*, 1965; D'Alonzo *et al.*, 1982) but little information about the *in vivo* effects of aromatic monocarboxylic acids is available (Tang *et al.*, 1968; Fuller *et al.*, 1971) and it is reduced mainly to two of them, 2,4-dichlorophenoxyacetic acid (Bucher, 1946), and anthracene-9-carboxylic acid (9-AC), one of the most potent myotonia-inducers (Palade and Barchi, 1977). With the purpose of increasing the knowledge about this acid, we have performed a series of studies about its *in vivo* acute and chronic effects (Villegas-Navarro, 1977; Morales-Aguilera *et al.*, 1983) and in this study we determined its pharmacokinetics. When the plasma data of 9-AC were fitted by non-linear regression analysis, an excellent agreement with an open two-compartment model was found.

Materials

The following drugs were used: Anthracene-9-carboxylic acid, Aldrich Chemical Co, U.S.A.; Chloroform, Sodium Bicarbonate and Sodium Hydroxide, J. T. Baker, U.S.A.; Heparin and Xylol, Sigma Chemical Co, U.S.A.; KY Jelly, Johnson and Johnson, U.S.A.; Polyethylene tubing, Des Var, U.S.A.; Foley's catheter No. 8 Adex, México.

The following apparatuses were used: Centrifuge, International Equipment Co, Model CS; Microcentrifuge, SOLBAT, Model H-07; Spectrophotofluorometer, Aminco-Bowman™, JA-8202G; pH Meter, Coleman Instruments, Model 37-A; Ultrasonic Cleaner, Tank Unit; Computer, VAX, Model 780.

Methods

A complete analytical procedure for the measurement of 9-AC in plasma, urine and whole blood was developed and has been published elsewhere (Villegas-Navarro *et al.*, 1983). In a summarized form, the procedure is a follows: *Extraction procedure.* To 1 ml of plasma or urine (diluted 1:10) was added 0.5 ml of 3M HCl, 0.5 g of sodium chloride and 50 ml of chloroform in an Erlenmeyer flask. The contents were ultrasonicated for 15 min, and centrifugated to separate the immiscible liquids. *Spectrofluorometric determination.* The spectra of excitation and emission in chloroform were obtained in an Aminco Bowman spectrophotofluorometer. The excitation spectrum exhibits a clear absorption peak at 355 nm. This wavelength was used subsequently for excitation of the samples. The fluorescence spectrum manifests an enhanced emission at 470 nm, and the peak at this wavelength was used to quantify 9-AC in plasma and urine *versus* standard. This procedure permits the total recovery of 9-AC from plasma and urine: plasma: 98 ± 1 %; urine: 101 ± 2 %.

Distribution of 9-AC between plasma and packed blood cells

The distribution of 9-AC between plasma and packed blood cells was quantified in the following manner: 0.2 ml of a solution of 9-AC (concentrations: 9.0×10^{-3}; 4.5×10^{-3}; 2.25×10^{-3}; 1.12×10^{-3} and 5.6×10^{-4} M) that had from 350 to 400 mOsm, was added to a sample of 3.8 ml of whole blood drawn from New Zealand White male rabbits. Final microhematocrit values were determined for each sample. The final concentrations of 9-AC in the blood were: 4.5×10^{-4}; 2.3×10^{-4}; 1.13×10^{-4}; 5.63×10^{-5} and 2.8×10^{-5} M. The mixture was kept at room temperature

during 30 min and then centrifuged. The equilibration time (30 min) was chosen since in control experiments it was found that equilibrium is reached in less than 10 min. The quantity of 9-AC in plasma was spectrofluorometrically determined after extraction with chloroform (Villegas-Navarro *et al.*, 1983) and the quantity in the packed cells was calculated as the difference between the quantity added to whole blood and the quantity recovered from plasma.

Pharmacokinetic studies. One hundred mg of 9-AC were placed in a 50 ml volumetric flask; 1.5 g of sodium bicarbonate were added with a few drops of sodium hydroxide for adjustment to pH 9 and the volume was made up with distilled water. The solution was ultrasonicated during 10 min to ensure the complete dissolution of 9-AC. It had 350 mOsm and was used for intravenous injection at a dose of $36\,\mu M \cdot kg^{-1}$ to unanesthetized New Zealand White male rabbits (2.5 to 3.2 kg). Three of these rabbits were injected and let free. They were observed during 3 h for myotonic signs. The time for onset and dissappearance of these signs and the response of the muscles to percussion were recorded. Another 13 rabbits were held individually in a cage which allowed no body movements but left the head free. One marginal ear vein was cannulated with fine polyethylene tubing and was used for the administration of heparin ($500\,IU \cdot kg^{-1}$), for the administration of 9-AC and for volume replacement (equal volume of iso-osmotic saline for each blood sample). The edge of the other ear was shaved and the marginal vein cut with a sharp razor at a point of convergence of arteriovenous anastomoses, which are aboundant in the rabbit's ear. The bleeding from the cut was controlled applying a small piece of cotton wool and a light clip. The ears were gently heated with a lamp between sampling. Thirty sec before sampling, the clip and cotton wool were removed and the ear was rubbed with xylol. The first 1 or 2 drops were discarded and sample volume was drawn within 50 sec. The urinary bladder was catheterized with a Foley's catheter lubricated with KY Jelly. During the urine collection periods the catheter was allowed to drain into a narrow-necked flask. The bladder was emptied as completely as possible by suprapubic pressure, the remaining urine was then removed by two instillations of 5 ml of sterile water. After the last rinse, 5 ml of air were introduced into the bladder and expelled again in order to remove the remainder of the wash-out fluid. The final volume thus collected was corrected in order to have the actual amount of urine. Control samples were taken at 1 min (for blood) and 5 min (for urine), before administration of 9-AC (time zero) to determine the absence of any fluorescent material at the wavelength used in the analytical procedure. Blood samples were taken starting 20 sec before 1, 5, 10, 20, 40, 60, 90 and 120 min after the administration of 9-AC. Urine samples were taken at 5, 10, 20, 35, 50, 70, 100, and 130 min after drug. In 4 of the rabbits (sham experiments), microhematocrit, plasma protein concentration (determined

by the method of Gornal *et al.*, 1948), and time for the drawing of the sample, were measured for each sample. In the other 9 rabbits 9-AC concentration was determined from the samples.

Statistical methods and mathematical treatment: Standard statistical methods (regression and variance analysis), with a probability level $p < 0.01$ for significance, were used (Snedecor and Cochran, 1978). A non-linear least-square regression analysis (program B M D P 3 R from the Department of Mathematics of University of California, Los Angeles) and the mathematical treatment of Riegelman *et al.* (1968), for an open two compartment model were also used.

Results

Myotonic signs in the rabbit. In the three animals let free after injection of 9-AC, the following pattern was observed within the first 90 min: 30 to 80 sec after the beginning of injection the animal presented spontaneous myotonic contractions. These contractions continued spontaneously or were elicited by percussion of a muscle or by lifting the rabbit and releasing it suddenly from a height of 10 to 20 cm. After being released the rabbit showed great difficulty in maintaining its normal position and remained immobile in a corner of the room. When the rabbit was stimulated, myotonic contractions appeared, with the 4 limbs in extension. The appearance of spontaneous myotonia in the anterior limbs preceded that in the posterior ones. The intensity of the myotonic phenomena gradually diminished and after 90 min totally disappeared. There were no signs of pain or disturbances of the central or of the autonomic nervous system.

Distribution of 9-AC between plasma and packed blood cells. Table I shows the percent of 9-AC recovered from plasma obtained from whole blood additioned with 9-AC at different concentrations (see methods) as well as microhematocrit values as mean $\pm$ SEM. The plasma percentage is approximately 78 and the quantity of 9-AC remaining in the packed cells is calculated as 22 %. The range of tested concentrations of 9-AC was 2.8×10^{-5} M to 4.5×10^{-4} M. At the highest concentration of 9-AC (4.5×10^{-4} M) the percentage recovered from plasma diminished to 72.4. The range of tested concentrations cover the ED_{50} *in vitro* (Palade and Barchi, 1977) and also the maximal concentration found in this kinetic study (2.0×10^{-4} M).

Hematocrit and plasma proteins concentration changes throughout the experiments. In order to evaluate the influence of the sampling procedure and of the time consumed by the drawing of each sample on the kinetic study, microhematocrit and plasma protein concentration were determined in 4 sham experiments. The drawing time for each of the 9 samples *per*

TAB

Distribution of 9-AC between

Conc. of 9-AC in whole blood (M)	4.5×10^{-4}	2.3×10^{-4}
Quantity of 9-AC recovered from plasma (%) (1)	72.4 ± 0.6	78.7 ± 0.8

Hematocrit = 39 ± 0.7.
(1) mean $\pm$ SEM; n = 10.

TABLE I

tween plasma and packed blood cells

^{4}	1.13×10^{-4}	5.63×10^{-5}	2.8×10^{-5}
	78.1 ± 0.7	78.5 ± 1.3	78.3 ± 0.7

Plasma concentration of 9-AC (μg/ml) at dif

Experiment Number	Weight Kg	1	5	
1	2.6	48.9	30.8	
2	2.6	35.9	–	
3	2.8	33.6	27.1	
4	2.4	42.3	28.0	
5	3.2	39.5	21.1	
6	2.7	–	36.0	
7	2.8	45.6	33.0	
8	2.8	54.8	–	
9	3.0	41.8	41.0	
$\bar{x} \pm$ SEM	2.8 ± 0.7	42.8 ± 2.2	31.0 ± 3.1	2

TABLE II

at different times after intravenous injection of 36 $\mu M \cdot kg^{-1}$

Minutes					
10	20	40	60	90	120
–	–	15.3	9.7	6.6	5.0
24.8	20.9	13.5	10.7	5.2	3.5
24.0	23.5	17.9	8.4	6.5	4.9
19.3	15.0	10.2	7.4	6.1	2.9
17.0	14.9	11.7	8.8	8.0	4.0
28.4	20.2	18.0	8.5	4.7	3.7
29.0	18.7	15.6	15.0	10.9	4.6
–	30.1	24.0	11.7	9.9	6.2
–	31.6	21.1	16.7	12.8	7.8
23.8 ± 1.7	21.9 ± 2.2	15.9 ± 1.3	10.7 ± 0.9	7.8 ± 0.9	4.7 ± 0.5

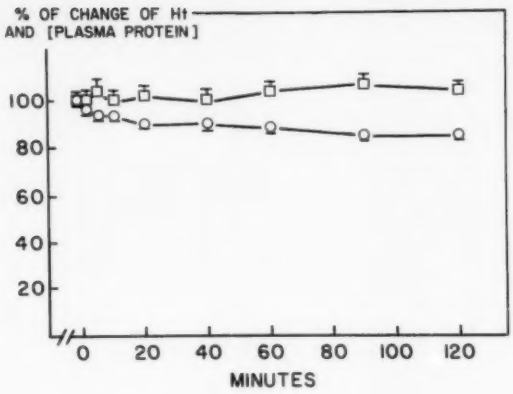

FIG. 1

Percentual changes in plasma protein concentration (□) and hematocrit (○) during the course of the sham experiments. Each point represents the mean ± SEM of 4 experiments. As 100 % was taken the value of hematocrit and of plasma protein concentration one minute before injection at time zero.

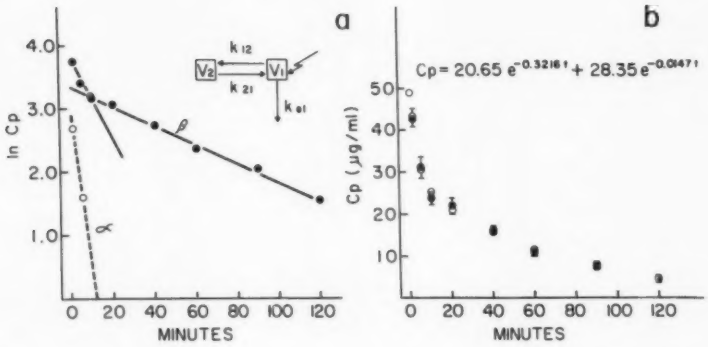

FIG. 2

Inset *a* shows the *ln* of 9-AC plasma concentration (C_p) against time after injection (36 $\mu M \cdot kg^{-1}$). Solid lines are regression lines. The dashed line was found by "feathering". Inset *b* shows the mean of the experimental values ± SEM (for n, see Table II), solid circles (●). Open circles (○) are the values calculated by means of the equation also displayed.

TA

Pharmacokinetic para

Dose (μM)	A ($\mu g \cdot ml^{-1}$)	B ($\mu g \cdot ml^{-1}$)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	AUC $\mu g \cdot min$
115	26.3	19.4	1.7	57.2	1671
87	30.0	19.6	2.7	43.0	1361
101	6.3	29.8	1.7	42.7	1859
94	32.9	24.1	2.5	50.9	1892
97	21.8	30.2	3.6	37.4	1751
101	18.7	27.6	4.7	53.7	2268
101	20.0	39.6	2.9	43.8	2592
108	5.6	39.6	5.0(1)	43.8	2627
94	10.1	29.1	2.2	38.7	1660
	19.1	28.8	2.80	45.7	1920
	±3.3	±2.4	±0.3	±2.2	±125

The last row depicts mean values ± SEM.
(1) This value was excluded from the calculation of the mean.

TABLE III

ic parameters for 9 experiments

AUC	K_{12}	K_{21}	$t_{1/2el}$	V_1	V_{dss}
$\cdot min \cdot ml^{-1}$	(min^{-1})	(min^{-1})	(min)	$(ml \cdot kg^{-1})$	$(ml \cdot kg^{-1})$
1671.7	0.200	0.171	26.6	187.2	379.0
1361.4	0.121	0.109	18.6	158.3	333.8
1859.2	0.064	0.320	35.3	221.7	265.6
1892.6	0.013	0.124	23.0	140.2	291.8
1751.2	0.060	0.117	23.3	153.8	232.3
2268.1	0.047	0.093	33.9	172.7	259.2
2592.0	0.066	0.161	30.1	134.1	189.0
2627.5	0.003	0.042	40.0	177.2	250.2
1660.3	0.070	0.239	29.3	204.2	264.7
1920.0	0.085	0.162	28.84	172.1	273.3
±125.0	±0.01	±0.020	±2.2	±9.7	±18.6

experiments was 50 ± 1.2 sec (mean $\pm$ SEM; $n = 35$). Plasma protein concentration did not overtly vary throughout the experiment as shown in Fig. 1. Hematocrit values did vary following the pattern also displayed in Fig. 1, with a maximal total decrease of about 15 % after the 9th sample.

Plasma values of 9-AC and choice of the compartmental model. Table II shows the plasma concentration values of 9-AC at the sampling times. A non linear least-square regression analysis (program B M D P 3 R; Department of Mathematics, University of California, Los Angeles) was applied to the data. The equation

$$C_p = 20.65e^{-0.3216t} + 28.35e^{-0.0147t} \qquad (i)$$

C_p = Plasma concentration at time t

was found by the computer program as the best fit for the mean values of all the experiments. Fig. 2*a* presents the *ln* of the experimental mean concentrations of 9-AC as well as the regression lines for those values obtained by "feathering". Fig. 2*b* depicts the experimental values and compares them with the values predicted by equation (i). The agreement is a good one and, since 9-AC was administered as an intravenous bolus, it was decided that the most adequate pharmacokinetic model for 9-AC is an open two-compartment mammilary model.

Pharmacokinetic parameters. Table III shows the pharmacokinetic parameters calculated by means of equation (i) derived from a mammilary two-compartment model (Riegelman *et al.*, 1968) for each of the

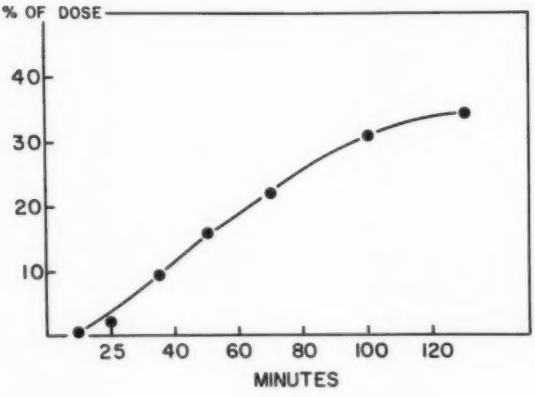

FIG. 3

Cumulative urinary excretion of 9-AC (spectrofluorometrically determined) as per cent of administered dose.

9 experiments plus their mean ± SEM. The steady state, as defined by Riggs (1963), is reached at minute 9.0. The mean steady state distribution volume per kg ($Vd_{ss} \cdot kg^{-1}$) was 273.3 ± 18.6 ml $\cdot kg^{-1}$.

Urinary excretion of 9-AC. Fig. 3 shows that the total amount of drug excreted unchanged in the urine (Au^{130}) in 130 min is 33.7 ± 5.2 % of the administered dose. On the other hand, we determined the value of f (the fraction of the drug present in the systemic circulation which is excreted unchanged in the urine) as

$$f = \frac{Au^{130}}{AUC_0^{120} \cdot V_1 \cdot K_{el}} = 0.38$$

(Wagner, 1971). This fraction corresponds very closely (within the experimental error) with the above value of 33.7 % of the total dose since in our experiments all the administered drug was injected into the circulation.

Discussion

Myotonic signs in the rabbit. The myotonic syndrome induced by 9-AC in rabbits is very similar to that described in goats (Bryant and Morales-Aguilera, 1971), rats (Nickolson *et al.*, 1975) and mice (Sethi, 1976). An apparent difference is that in the rabbit the disappearance of the overt myotonic signs is gradual and seems to be complete in 90 min, whereas in the goat the myotonia induced by the same dose (36 $\mu M \cdot kg^{-1}$) disappears almost suddenly at minute 70 (Bryant and Morales-Aguilera, 1971).

Distribution of 9-AC between plasma and blood cells. There are no published values of the distribution of 9-AC between plasma and blood cells. Therefore, it was considered convenient to establish the role of the blood cells on the disappearance of 9-AC from plasma. It was found that the concentration of 9-AC in plasma is approximately two times higher than in the packed cells (volume/volume). *i.e.*, only 22 % of the total amount of 9-AC in blood is found in the packed cells (when the values are corrected according to hematocrit). The per cent distribution of 9-AC between plasma and packet cells does not change within the range of concentrations of 9-AC found in the kinetic experiments (5 to 43 $\mu g \cdot ml^{-1}$), and the time to reach equilibrium between plasma and cells is less than 10 minutes. The kinetic analysis indicates that 9-AC distribution fits a two compartment model with a time for equilibrium at the steady state being reached in 9 min, and since the time to equilibrium between plasma and blood cells is less than 10 min, this equilibrium occurs within the range of time in which the first compartment is predominantly manifest.

Sampling procedure. The total time for the drawing of each sample

(50 sec on the average) and the beginning of each collection period (20 sec before the nominal time) assure us that the samples are a fair approximation to the "true" value of the 9-AC concentration at the nominal times. On the other hand, the variance analysis of the concentration values from Table II indicated that the variance between the means of the different samples is homogeneous, *i.e.*, random factors affected similary all samples (Ostle, 1963).

The blood drawn from each rabbit during the whole experiment was within 17 % of the total estimated blood volume (mean = 57.3 $ml \cdot kg^{-1}$; Armin *et al.*, 1952). The blood was drawn in 3 ml samples up to a total of 27 ml (9 $ml \cdot kg^{-1}$) over a period of 120 min. The general consensus (Chien, 1958; Guyton, 1961; Zimpfer *et al.*, 1982) is that this type of bleeding does not introduce significant hemodynamic changes (blood volume, arterial pressure, heart rate or circulation time). Nevertheless, we tried to estimate the amount of hemodilution by means of the changes in hematocrit and protein plasma concentration. Plasma protein concentration did not significantly vary in the sham experiments (Fig. 1). This finding confirms that protein replenishment occurs during small bleedings and that the vascular protein content rapidly returns to normal (Cope and Litwin, 1962). This replenishment is predominantly the result of a net transfer of pre-formed protein from the extravascular compartment into the blood and becomes apparent in a few min (Katz *et al.*, 1970; Yuile *et al.*, 1959). On the other hand, hematocrit values decreased following the pattern depicted in Fig. 1. The accumulated decrease at the end of 120 min (15 %) suggests an average hemodilution factor of 1 % to 2 % between two consecutive samples. This hemodilution, which includes the dilution due to the replacement of the drawn samples with isotonic saline (see methods), would explain the fall in hematocrit in our experiment but is not sufficient to produce any significant change in the hemodynamic properties of the circulatory system (Chien, 1971) or to alter the plasma kinetic analysis.

Choice of the compartmental model and pharmacokinetic parameters. The proposed two compartment mammilary model is the simplest model required to fit the present data and the model gives a good agreement between the raw data and the predicted values. This agreement is apparent in Fig. 2. Compartment one represents the site into which 9-AC was injected and from which blood samples were taken. Its volume (V_1) is equal to 172.1 ± 9.7 $ml \cdot kg^{-1}$. This volume is about three times larger than the vascular space (57.3 $ml \cdot kg^{-1}$), but it is smaller than the total extracellular space estimated by Harrison *et al.* (1936) as 246 $ml \cdot kg^{-1}$. The mean distribution volume per kg at steady state between the two compartment ($Vd_{ss} \cdot kg^{-1}$) is 273.3 ± 18.6 $ml \cdot kg^{-1}$ and corresponds to 27.3 % of the body weight. The difference between $Vd_{ss} \cdot kg^{-1}$ and the extracellular volume $\cdot kg^{-1}$ is small (*ca.* 10 %). These facts plus the almost immediate appearance of myotonia (30 to 80 sec) suggest that in the first 9 min 9-AC

passes the capillary walls and reaches the tissues but probably does not penetrate into the cells to a significant extent.

Excretion and fate of 9-AC. The drug determined in the urine was 9-AC without change since with our spectrofluorometric method (Villegas-Navarro *et al.*, 1983) the drug is determined by means of both the emission and absorption spectra. The close correspondence between the percent of drug eliminated in the urine and the value of f means that, within the time of our experiments (130 min), approximately 65 % of the injected 9-AC remains in a body reservoir or it is biotransformed. On the other hand, although to our knowledge there are not studies of 9-AC biotransformation, it is known that other aromatic monocarboxylic acids are biotransformed mainly to conjugates (Møller and Sheikh, 1983). For instance, benzoic acid and several methylenedioxyphenyl monocarboxylic acids (Klungsøyr and Scheline, 1981) conjugate mainly with glycine (Bray *et al.*, 1955). Furthermore, another myotonic agent, clofibric acid, is excreted mainly as glucuronate (Azarnoff *et al.*, 1965). Thus, there are good reasons to believe that 9-AC may be eliminated as a conjugate.

The relation between 9-AC pharmacokinetic and its site of action. The comparison between the plasma molar concentrations of 9-AC found in this study as capable of inducing myotonia *in vivo* and the molar concentrations of 9-AC that Palade and Barchi (1977) found as causing myotonia *in vitro* is also relevant to the point. In their study, those authors also quantified the myotonic state as a decrease in sarcolemmal chloride conductance. The results of the comparison we propound are shown in Table IV.

The values on the left side were calculated on the basis of our pharmacokinetic constants while those on the right side were calculated from the study by Palade and Barchi. As a part of the interaction between 9-AC and the sarcolemma, Bryant and Morales-Aguilera (1971) on the basis of their *in vitro* study on goat intercostal muscle postulated that the 9-AC molecule anchors itself to the chloride channel in the sarcolemma by means of hydrophobic (i.e., "weak") forces. Palade and Barchi (1977) supported that assertion and even found that the potency of a whole series of monocarboxylic aromatic acids (benzoic acid derivatives including 9-AC) was directly proportional to the log P (partition coefficient) of each compound, that is, the degree of chloride conductance blockade was directly related to the hydrophobicity of the compound. The *in vitro* experiments of Palade and Barchi made also clear that the site of action is in the sarcolemma. Therefore, the correspondence between the molarities of 9-AC that are active or inactive *in vivo* and *in vitro* is not surprising and the kinetic behavior of 9-AC found in this study also suggests that the plasma concentration of 9-AC reflects its concentration at the site of action (muscle membrane). The fact that a concentration of 1×10^{-5} M, which is able to reduce chloride conductance down to 50 % of the control values *in vitro*,

T

Comparison between concentrat

$t_{1/2el}$	Minutes after injection	Plasma concentration *in vivo*
$t_{1/2el}$	28.8	1.07×10^{-4} M
$3t_{1/2el}$	86.5	2.69×10^{-5} M
$4t_{1/2el}$	115.3	1.34×10^{-5} M
$5t_{1/2el}$	144.2	6.74×10^{-6} M

([1]) From Palade and Barchi, 1977.

TABLE IV

centrations of 9-AC active in vivo and in vitro

ration	Myotonia *in vivo*	In vitro concentration (1)	Per cent reduction of Cl^- conductance (1)
M	Present	1.0×10^{-4} M	100
M	Present	3.4×10^{-5} M	75
M	Doubtful	1.1×10^{-5} M	50
M	Absent	5.0×10^{-6} M	20

renders only doubtful results *in vivo* is also in favor of the above interpretation since it could be explained by the analysis of Barchi (1975), who by computer simulation has shown that it is necessary to reduce the chloride conductance below 30 % of its normal value before producing a myotonic state of the normal muscle.

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Interaction of Benznidazole Reactive Metabolites with Rat Liver Deoxyribonucleic Acid and Nuclear Proteins

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Abstract—Benznidazole (Bz) (N-benzyl-2-nitro-1-imidazole acetamide) is one of the drugs used in the chemotherapy of Chagas' disease. Microsomal suspensions anaerobically activated Bz in the presence or absence of NADPH to metabolites that covalently bind to proteins or to DNA incorporated into the incubation mixture. At high Bz concentrations (0.2 mM) NADPH enhanced the intensity of the process while at low Bz concentrations (0.02 mM) it decreased it. Nuclear preparations were also able to anaerobically activate Bz to reactive metabolites that bind covalently their DNA and proteins. The process was enzymatic and required NADPH. Most of the interaction between Bz metabolites and nuclear proteins involved the acidic non-histone proteins and those from the nuclear sap. A minor part of the interaction was with basic deoxyribonucleoproteins, acidic ribonucleoproteins and residual fractions. Almost no interaction with histones was observed. Potential toxicological implications of the observed interactions are analyzed.

Introduction

Previous studies from other laboratories reported mutagenic effects of Benznidazole (Bz) on S. thyphimurium (Nagel and Nepomnaschy, 1983) and on Klebsiella pneumoniae (Voogd, 1981). Recent studies by Masana *et al.* (1984) in our laboratory gave evidence of the formation of reactive metabolites in anaerobic incubation mixtures containing Bz, liver microsomes and NADPH. Moreover, covalent interactions of Bz reactive metabolites with proteins from different tissues of female rats and the fetus were observed, when Bz was orally administered to pregnant animals

(Toranzo *et al.*, 1984). In this work, we report our studies directed to test whether reactive intermediates arisen during reductive Bz metabolism, are able to interact with liver DNA and nuclear proteins *in vitro*.

Results might be important to understand the mutagenic effects of Bz described above and in relation to possible risks derived from the widespread use of Bz in the treatment of Chagas' disease (Boainain, 1979; Carpintero, 1983), a parasitic disease affecting several million people in Latin-America (WHO, 1981).

Methods

Chemicals: Benznidazole (Bz) (N-benzyl-2-nitro-1-imidazole acetamide) with ^{14}C in position 2 of the imidazole ring (sp. act. 58.6 μCi/mg) was a gift from Hoffmann La Roche, Switzerland, no detectable labeled impurities were found in the sample of ^{14}C-Bz employed in this study when analyzed by silica gel TCL using chloroform-ethanol (9:1) as solvent. Bovine Thymus DNA was purchased from Sigma Chemical Co. Other chemicals employed were of the best quality available.

Animals: Sprague-Dawley male rats (200–220 g) were used in this studies. The animals were fasted 12–14 hr before use but they has access to water ad lib.

The animals were killed by decapitation and bled, and their livers were rapidly excised and processed to obtain the microsomal or nuclear preparations.

Procedure: Fresh microsomal fractions were prepared every day from livers homogenized in a teflon glass Potter-Elvehjem homogeneizer with 4 vol of 1.15 % KCl. The homogenate was centrifuged for 20 min at 9,000 *g* and the supernatant fraction was further centrifuged for 1 hr at 105,000 *g* to obtain microsomal pellets.

Fresh Nuclear Preparations were obtained every day by the procedure of Blobel and Potter (1966) and further purified by two additional resuspensions in the medium used by the former authors and centrifugation for 10 min at 800 g. These further purified nuclear preparations regularly gives fully preserved nuclei when observed by phase contrast microscopy and only traces of contamination with endoplasmic reticulum when checked by electron microscopy.

Incubation Mixtures: Incubation mixtures contained rat liver microsomes (4–5.5 mg/ml microsomal protein per ml of incubation mixture), 80 μl of NADPH generating system and 3 μl of a dimethyl formamide solution of ^{14}C-Bz (0.02 mM) and 20 mM phosphate buffer pH 7.4, to a final volume of 5 ml). When rat liver nuclei were used, suspension media consisted of 0.25 M sucrose in 50 mM Tris/HCl buffer pH 8.0 containing 1 mM EDTA and 5 mM mercaptoethanol. This medium is essential for the stability of

the nuclear preparations in the incubation mixtures. Final concentration of ^{14}C-Bz and NADPH was similar to that employed for experiments with microsomes. Nuclear protein concentrations were about 3.3 mg per ml of incubation mixture.

Mixtures were incubated for 60 min at 37° C under an anaerobic (oxygen-free nitrogen) or under an air atmosphere when indicated. After the incubation period, samples were pelleted at 105,000 *g* for 60 min and the supernatant fraction saved for studies on covalent binding to DNA. Pellets were treated with TCA for determination of covalent binding to proteins. The NADPH-generating system used was: 20 mM phosphate buffer pH 7.4, 0.4 ml; 1 M $MgCl_2$, 0.1 ml; isocitric acid dehydrogenase type I from porcine heart (Sigma Chemical Co), 0.3 ml; DL-isocitric acid trisodium salt, 62 mg; NADP sodium salt, 10 mg.

Covalent Binding of ^{14}C-Bz reactive metabolites to proteins: The determination of the extent of the covalent binding of Bz metabolites to microsomal proteins was performed as described by Díaz Gómez *et al.* (1973). The TCA pelleted proteins were washed three times with 5 % TCA, heated with 5 % TCA for 30 min at 85° C–90° C to eliminate nucleic acids and finally washed successively (until radioactivity in washings was not detectable) with ethanol-diethyl ether-chloroform (2:2:1), acetone and diethyl ether. These washings remove the lipids and the unbound drug and dry the precipitate. An aliquot of the protein sample was dissolved in concentrated formic acid and counted by liquid scintillation.

Covalent Binding of ^{14}C-Bz reactive metabolites to DNA: In studies where DNA (2 mg DNA/ml) was incorporated into incubation mixtures containing NADPH generating system, ^{14}C-Bz and microsomes, the isolation of labeled DNA was accomplished by the phenol-cresol procedure as described by Swann and Magee, 1968. The purified DNA obtained was dried, hydrolyzed by heating in 2 ml 1 N hydrochloric acid for 60 min at 100° C, evaporated to dryness at 50° C and taken up in 0.05 ml of water, 0.5 Protosol (New England Nuclear) and 10 ml Dimilume (a liquid Scintillation cocktail including a chemoluminiscence inhibitor manufactured by Packard) and counted by liquid scintillation.

The isolation of DNA from purified liver nuclei was accomplished by the procedure described by Vaught and Bresnick (1976). After incubation the nuclei were pelleted by centrifugation for 10 min at 1,000 *g*, washed three times and lysed in 0.03 M NaCl, 3 mM Nacitrate, 2 % Na dodecyl sulfate (SDS) and the solution was extracted with buffer-saturated phenol. The phenol phase was saved for the determination of protein binding while the nucleic acids were precipitated from the aqueous fraction with ethanol. The nucleic acid precipitate was redissolved in buffer, 200 μg preheated RNAse (80° C for 10 min) was added and digestion of the RNA allowed to continue for 60 min at 37° C. The DNA was then precipitated with ethanol, washed twice with ether, and hydrolyzed and processed as described above.

Separation of the different nuclear protein fractions from purified nuclear preparations was performed by the procedure described by Steele and Busch (1963). To improve the quantitative precipitation by TCA of all the label associated with the proteins in fraction I to V of Steele and Busch, pure egg albumin was added to each fraction. All the protein in each fraction was then purified, dried, and counted as described above for microsomal proteins.

Results

Interaction of Benznidazole (Bz) reactive metabolites with microsomal proteins and DNA incorporated into the incubation mixtures

When liver microsomes were incubated Bz in the presence or absence of NADPH, reactive metabolites were formed that bound covalently to microsomal proteins or to DNA added to the incubation mixture (Table I). The response to NADPH addition however, depended on Bz concentration. At Bz concentrations of 0.2 mM, NADPH intensified covalent interactions of Bz reactive metabolites with both, microsomal proteins and DNA (Table I). In contrast, when Bz concentration was lowered to 0.02 mM, NADPH addition resulted in decreases in the intensity of the

TABLE I

Interaction of Benznidazole (Bz) reactive metabolites with microsomal proteins and DNA incorporated into the incubation mixtures

	Bz Covalently bound	
Experimental Condition*	pmol/mg mic. protein	pmol/mg DNA
Bz 0.2 mM		
No NADPH	84.80	162.15
NADPH	218.85	207.82
Bz 0.02 mM		
No NADPH	252.01	381.81
NADPH	113.76	100.16

* Incubation mixtures contain microsomes (4–5 mg/ml microsomal protein) ^{14}C-Bz 0.02 mM or 0.2 mM as indicated and DNA (2 mg/ml). When indicated NADPH generating system was added. The data represent the average of two simultaneous determinations. Values at each concentration of Bz are representative of three duplicate determinations but results at different concentrations of Bz were not obtained using the same microsomal preparation so they can not be compared each other. They are only relevant in observing the effect of NADPH addition at each concentration.

TABLE II

Nuclear activation of Benznidazole (Bz) to reactive metabolites that bind covalently to DNA and nuclear proteins

Experimental Condition*	Bz Covalently bound	
	pmoles/mg protein	pmoles/mg DNA
No NADPH	2.87	2.65
NADPH	19.82	17.03

* Incubation mixtures contain nuclei and ^{14}C-Bz 0.02 mM and they were performed under N_2. When indicated NADPH generating system was added. The data represent the average of two simultaneous determinations.

covalent binding of Bz metabolites to both DNA and microsomal proteins (Table I). Lowering of Bz concentrations in the absence of NADPH, was accompanied by a marked preference of Bz reactive metabolites to covalently bind to DNA in relation to microsomal proteins (Table I). Heating of the microsomal preparation for 5 min at 100° C completely destroyed the ability of liver microsomes for Bz activation both in the presence of absence of NADPH.

Nuclear activation of Benznidazole (Bz) to reactive metabolites that bind covalently to DNA and nuclear proteins

Nuclear preparations were able to anaerobically activate Bz in the presence of NADPH to reactive metabolites that bound covalently to DNA and nuclear proteins (Table II). The process proceeded only to a minor

TABLE III

Nuclear activation of Benznidazole (Bz) to reactive metabolites that bind covalently to nuclear proteins

Experimental Condition*	Bz Covalently bound (pmoles/mg protein)
No NADPH (Air)	5.39
No NADPH (N_2)	2.39
NADPH (Air)	6.64
NADPH (N_2)	33.84
NADPH (N_2 + heating)	2.74

* Incubation mixtures contain nuclei and ^{14}C-Bz 0.02 mM and they were performed under air or N_2 as indicated. When indicated NADPH generating system was added. Heating was performed for 5 min at 100° C. The data represent the average of two determinations.

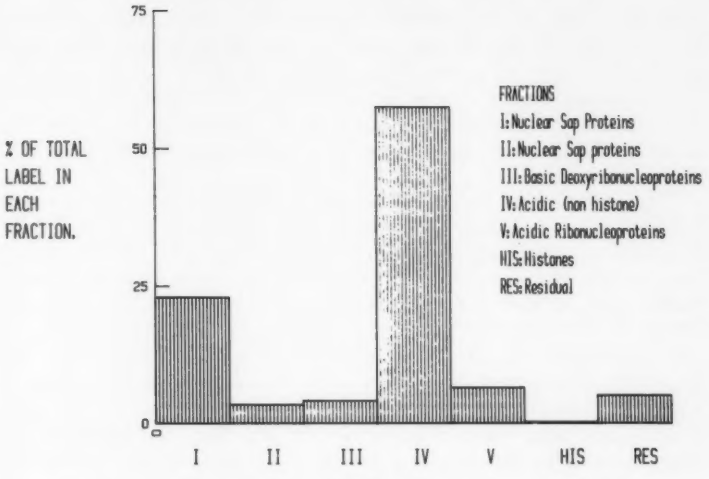

FIG. 1

Nuclear protein fractions involved in the interactions with Benznidazole (Bz) reactive metabolites. The pattern of distribution of label associated with each different nuclear protein fraction is depicted. The non-histone protein fraction is the one interacting more intensively.

extent under an oxygen atmosphere or when NADPH was excluded and it was almost zero when nuclear preparations were heated (Table III).

Nuclear protein fractions involved in the interaction with Benznidazole (Bz) reactive metabolites

Most of the interaction between Bz reactive metabolites produced during nuclear anaerobic activation of Bz in the presence of NADPH and nuclear proteins involved the acidic non-histone fraction (Fig. 1). A significant part of the interaction, occurred with fraction I of the nuclear sap proteins and a minor part involved the nuclear proteins in fractions II, III, V and residual of the Steele and Bush (1963) fractionation procedure (Fig. 1). Almost no interaction with histone proteins was observed (Fig. 1).

Discussion

Previous studies from our laboratory, gave evidence that under anaerobic experimental conditions, liver microsomal preparations activate Bz to reactive metabolites that bind covalently to proteins and phospholipids (Masana *et al.*, 1984; Toranzo *et al.*, 1984). The present studies show that these Bz reactive intermediates are also able to interact

with DNA added to the incubation mixtures. This is not unexpected, since nitroderivatives are known to produce free radical intermediates when reduced (Hewick, 1982) and free radicals are known to interact with several cellular components, including proteins, phospholipids and DNA (Castro, 1984). Interestingly, DNA is a favorite target in relation to microsomal protein, for specific labeling in the former, is higher than in the latter.

We also performed studies on CB to DNA and protein in incubation mixtures where Bz concentration was lower than in the above described experiments, but closer to that potentially occurring *in vivo*, assuming uniform distribution of the drug in the whole body. Surprisingly, in these studies at lower concentrations, the behavior of the Bz activation system changed significantly. Indeed, in this case, addition of NADPH resulted in decreases in the intensity of the CB of Bz metabolites to macromolecules rather than increases, as it was observed when Bz concentrations were high. These results suggest the existence of at least two Bz activation systems, one being NADPH-dependent and the other which is not. The system not depending on the presence of NADPH would have more affinity for Bz and would be saturated at lower concentrations than the one which requires NADPH. These characteristics would explain why the NADPH-independent activation system, preferentially operates at low Bz concentrations, while the NADPH requiring pathway would be the one predominating at higher Bz concentrations. We found particularly difficult to imagine what reactions would lead to the formation of a reactive metabolite in the absence of added NADPH as reducing agent. However, this behavior was repeatedly observed and it was not due to the presence of directly reacting impurities in our samples of ^{14}C-Bz. Indeed no such impurities were detectable and more important, heating inactivates this ability for Bz activation in the absence of NADPH thus revealing the enzymatic nature of the process. This susceptibility to heating was also observed in the case of the NADPH dependent pathway of Bz activation. The results here described also suggest the presence of a NADPH-dependent metabolic pathway not forming reactive metabolites. The existence of this detoxicating process, can clearly be seen at low Bz concentrations, when addition of NADPH to incubation mixtures decreases the intensity of the CB of Bz reactive metabolites to macromolecules.

In addition to the microsomal activating pathways, we also found that highly purified nuclear preparations, free of endoplasmic reticulum, were able to activate Bz to reactive metabolites interacting with both, nuclear DNA and proteins. This activating system is NADPH-dependent and enzymatic. The relevance of this nuclear activating pathway might rest on the increased chances for reactive metabolites formed at nuclear sites, to attack critical DNA and/or nuclear proteins, rather than on similar reaction products formed at distant sites in the endoplasmic reticulum as

previously described by our laboratory (Masana *et al.*, 1984). In fact, here we report that nuclear activation leads to metabolites reacting with both target macromolecules DNA and nuclear proteins. Nuclear protein fractionation studies revealed that label from ^{14}C-Bz reactive metabolites is almost exclusively associated with non-histone proteins.

The overall analysis of the above described studies bring interesting points and questions for analysis. Indeed, the interactions reported here of Bz metabolites with DNA might explain the mutagenic properties of this drug in the Ames test, reported by other workers (Nagel and Nepomnaschy, 1983). In addition, since most carcinogens interact covalently with DNA (Hemminki, 1983; Kubinsky *et al.*, 1981; O'Connor, 1981), are mutagenic (Ames *et al.*, 1975) and preferentially interact with the non-histone fraction of nuclear proteins (Gronow, 1980) as it happens with Bz, these results suggest the need for additional studies on the potential carcinogenic risk of Bz. In this line, we recently performed studies on micronucleus formation in bone marrow of mice treated with Bz (Gorla and Castro, 1985). We found that Bz, in contrast to Nifurtimox does not increase micronucleus formation under the experimental conditions tested (Gorla and Castro, 1985). The interactions of Bz reactive metabolites with DNA, proteins and phospholipids reported here and before (Masana *et al.*, 1984, 1985; Toranzo *et al.*, 1985) might also be involved in the acute toxic effects of Bz. Indeed, the CB of reactive metabolites or free radicals to cellular components is known to be involved in different expressions of cell injury (Castro, 1984). This hypothesis is enlightened by the findings that Bz, in contrast to other free radical producing chemicals, requires rather high concentrations to stimulate lipid peroxidation *in vitro* (Moreno *et al.*, 1982; Docampo and Moreno, 1984) and does not promote lipid peroxidation *in vivo* as measured by pentane evolution studies in rats (Castro and Castro, 1985).

Further work would be necessary to test this purely speculative hypothesis, suggesting the possible participation of the interactions described here for Bz reactive metabolites with DNA and nuclear proteins in deleterious effects of Bz and to understand the intriguing nature of the enzymatic process activating Bz in absence of NADPH.

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The Effect of Nifedipine on Norepinephrine Release

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Abstract—Recently, we reported that verapamil and diltiazem caused a marked release of ^3H from ^3H-norepinephrine labelled tissues with rich sympathetic innervation. In the present study, we investigated the effect of nifedipine in rat tail artery and guinea-pig vas deferens. *In vitro* superfusion of the arteries with nifedipine ($10^{-6}-10^{-4}$ M) caused a significant release of ^3H both in spontaneously hypertensive and in normotensive rats, particularly after the highest concentration of the drug. Release of ^3H induced by transmural stimulation (1 Hz, 2 msec, 10 V) was also significantly greater in vessels superfused with nifedipine than in controls. Column chromatographic analysis revealed that nifedipine did not alter the proportion of norepinephrine and its metabolites in spontaneous or electrical stimulation induced ^3H-overflow suggesting that it releases norepinephrine from sympathetic nerves intracellularly. Nifedipine ($10^{-9}-10^{-4}$ M) superfusion caused release of ^3H-norepinephrine also in the vas deferens of guinea-pig. The effect of nifedipine observed in the present study was similar but less pronounced than that described by us for verapamil and diltiazem. The results indicate that calcium entry blockers act on sympathetic nerves and that this effect is likely explained by an action other than inhibition of Ca^{2+} influx into the cells.

Introduction

The effect of calcium entry blockers on sympathetic nerves is not yet clarified. High concentrations of verapamil (Göthert *et al.*, 1979) and nifedipine (Starke and Schnemann, 1973) in rabbit heart, of diltiazem (Zelis *et al.*, 1982) in rabbit pulmonary artery and of D-600 in rat atrium

(Callanan and Keenan, 1984) were reported to inhibit electrical stimulation induced norepinephrine (NE) release. Other investigators, however, found that calcium antagonists were ineffective to interfere with the effect of nerve stimulation in cat and dog heart (Haeusler, 1972; Nayler and Szeto, 1972) or in rabbit artery (Kajiwara and Casteels, 1983). Our own experiments revealed that verapamil (Zsotér *et al.*, 1984a) and diltiazem (Zsotér and Wolchinsky, 1985) not only failed to inhibit ^3H release from ^3H-NE labelled tissues, but caused a pronounced increase in spontaneous and transmural stimulation induced NE overflow. Enhanced NE release was unexpected from calcium antagonistic drugs which inhibit Ca^{2+} influx into the cells but most recently it was reported also by other authors for verapamil, D-600, diltiazem and nicardipine (Chaudhry and Vohra, 1984a; Chaudhry and Vohra, 1984b; Karaki *et al.*, 1984; Takata and Kato, 1984).

The release of neuronal transmitter from adrenergic nerves may alter the integrity of baroceptor reflexes and thus profoundly modify the effect of calcium entry blockers on the heart and vessels. This was an additional reason that we decided to study the effect of another calcium channel blocker, namely of nifedipine, on NE release from tissues with rich sympathetic innervation, as the rat tail artery and the guinea-pig vas deferens.

Methods

Experiments in Rats

Seven to nine week old male spontaneously hypertensive rats (SHR) of the strain originally bred by Okamoto and Aoki and normotensive Wistar Kyoto rats (WKY) obtained from the Taconic Farms were used in these experiments. Systolic blood pressure measured on the tail of unanesthetized animals, as in our previous experiments (Zsotér *et al.*, 1982), was 118.1 ± 1.2 mm Hg in WKY and 169.8 ± 3.9 mm Hg in SHR.

The proximal part of the tail artery was removed from rats anaesthetized with ether, carefully cleaned, weighed and suspended in Krebs solution bubbled with 95 % O_2 and 5 % CO_2 at 37° C for 30 min. The solution had the following composition (in mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 2.6, $MgCl_2$ 1.2, NaH_2PO_4 1, glucose 11. Subsequently, the vessels were placed for 60 min in a tissue bath containing 10 μCi ^3H-NE (1-[7 ^3H]-NE, New England Nuclear; specific activity 17.2 Ci/mmol) in 5 ml Krebs solution; the concentration of NE was 1.16×10^{-7} M. During the incubation and superfusion, ascorbic acid (0.11 mmol) and Na EDTA (0.004 mmol) were added to the solution to delay the breakdown of NE. After labelling with ^3H-NE, the vessels were transferred to another tissue bath and superfused with the modified Krebs solution at 37° C for 180 min at the rate of 1 ml/min. When the effect of O-Ca^{2+} was studied, after

75 min of superfusion, Krebs solution was replaced by an identical one in which $CaCl_2$ was replaced by NaCl. The effluent from the artery was collected at 5 min intervals. The dead space between the tissue bath and the tube collecting the ^{3}H-overflow was about 2 ml.

For transmural stimulation we used platinum electrodes, placed parallel to the vessels. Square wave direct impulses (1 Hz, 10 V, 2 msec) were triggered by a Grass S44 stimulator at 60–65, 90–95, 120–125 and 150–155 min of the superfusion. The artery was perfused with nifedipine (Miles Laboratories, Toronto, Canada), 10^{-6} M at 80–110 min, 10^{-5} M at 110–140 min and 10^{-4} M at 140–180 min. In one series of experiments, no nifedipine was given during the superfusion. In order to measure the residual ^{3}H content in the arteries, the vessels were digested at 56° C for approximately 5 hr in Nuclear Chicago solubilizer (Amersham Corp., Illinois). Radioactivity was counted in each sample of the efflux and in the digested artery by Beckman 9000 Liquid Scintillation Counter. The results were expressed as DPM/mg wet weight of vessel and as fractional release, that is ^{3}H-efflux as a portion of the amount of ^{3}H in the tissue. Nifedipine or transmural stimulation induced changes in ^{3}H-overflow were calculated also as percentage changes of these values, for each 5 min periods compared to 5 min periods measured immediately prior superfusion with the given concentration of the drug or electrical stimulation.

Column chromatography as described by Graefe *et al.* (1973) was used like in our previous studies (Zsotér *et al.*, 1982) to separate NE from its metabolites in ^{3}H-overflow. In these experiments, one sample collected at 55–60 min (i.e. before transmural stimulation and nifedipine administration) and another one at 155–160 min of superfusion (i.e. after 10^{-4} M nifedipine) were analyzed for NE; normetanephrine (NMN); O-methylated deaminated products (OMDA), that is methoxy-hydroxy-phenylglycol (MOPEG) and methoxy-hydroxy-mandelic acid (VMA); dihydroxyphenylglycol (DOPEG) and dihydroxymandelic acid (DOMA) content.

For statistical analysis, *t*-tests for unpaired and paired samples and regression analysis were done as indicated in the text.

Experiments in Guinea-Pigs

Male, albino guinea-pigs were used in these experiments. One vas deferens was removed from each animal under ether anesthesia. The tissue was cleaned and incubated in 10 μCi ^{3}H-NE for 60 min and superfused as in the rat experiments. The collection of efflux from the tissue, stimulation with platinum electrodes at 60–65 min, 90–95 min, 120–125 min, 150–155 min and 185–190 min, as well as the measurement of ^{3}H in the samples and in digested vessels were performed as in the rats. In the guinea-pig, nifedipine was given, however, in different concentrations:

10^{-9} M at 85–115 min, 10^{-7} M at 115–145 min, 10^{-5} M at 145–180 min and 10^{-4} M at 180–210 min of superfusion.

Results

Experiments in Rats

Nifedipine enhanced ^{3}H-overflow from ^{3}H-NE labelled tail arteries in both WKY and SHR. In Table I results are expressed as percentage change of fractional release. Prior to nifedipine there was a decline in ^{3}H-efflux. This decline was replaced by an increase in ^{3}H-overflow in both WKY and SHR during perfusion with nifedipine. The difference was particularly obvious with the highest concentration, that is with 10^{-4} M. This effect of nifedipine did persist for approximately 20 min and therefore, Table I gives percentage changes for 20 min periods.When ^{3}H-overflow was expressed as DPM/mg vessel (and not as fractional release as in Table I), statistical analysis gave identical p values as for percentage changes shown in Table I. The effect of nifedipine on ^{3}H-overflow in WKY was dose related. When results, expressed as DPM/mg tissue, were subjected to regression analysis, the slope was 0.190 ± 0.055 demonstrating a significant correlation between the concentration and the effect of the drug ($p < 0.01$).

Transmural stimulation induced ^{3}H release was also enhanced by nifedipine. Table II demonstrates that electrical stimulation induced increment in ^{3}H-overflow, expressed as DPM/mg, repeated at 30 min intervals, remained similar in untreated arteries both in WKY and SHR. In contrast to this, in vessels superfused with increasing concentrations of nifedipine (10^{-6} to 10^{-4} M), the response to transmural stimulation, expressed as DPM/mg, became significantly greater than in untreated vessels. While mean values of ^{3}H-overflow at 60–70 min, i.e. prior superfusion with nifedipine were higher than in control animals, the difference was statistically not significant, and it became significant only after perfusion of the vessels with nifedipine. The effect of transmural stimulation, expressed as percentage increase of fractional release, was also significantly greater ($p < 0.02$) than in control WKY or SHR resp.

Although nifedipine (10^{-4} M) enhanced significantly stimulation induced ^{3}H-overflow from ^{3}H-NE labelled tail arteries of WKY and SHR, the effect was substantially less than in our previous experiments, during superfusion with 10^{-4} M verapamil or diltiazem (Fig. 1).

It is important to know whether nifedipine increases ^{3}H-overflow by releasing mainly ^{3}H-NE or its metabolites. Figure 2A gives the results of column chromatographic separation of NE from normetanephrine, O-methylated deamintated products, dihydroxyphenylglycol and dihydroxymandelic acid in 8 arteries of 4 WKY and 4 SHR, before and

Nifedipine in superfusate			0
		n	60–80 min
	WKY	4	−12.3 ± 4.1
	SHR	4	−26.8 ± 7.7

% change (mean + S.E.) of fractional release of ^3H.

Results are expressed as sum of % changes during 20 min periods (i. 55–60 min, 80–90 min, 115–120 min and 145–150 min, respectively.

▲ $p < 0.02$ compared to results at 60–80 min control period.

● $p < 0.01$ compared to results at 60–80 min control period.

▪ $p < 0.001$ compared to results at 60–80 min control period.

TABLE I

ine on ^{3}H-overflow from rat tail artery

0	10^{-6} M	10^{-5} M	10^{-4} M
0 min	90–110 min	120–140 min	150–170 min
3 ± 4.1	+19.7 ± 7.9●	+26.4 ± 8.7●	+114.9 + 2.4*
8 ± 7.7	+28.0 ± 9.2●	+6.9 ± 7.7▲	+96.6 ± 9.0*

iods (i.e. four 5 min collections) compared to preceding 5 min periods of superfusion at ely.

		n	60–70 mi
Control	WKY	6	353.7 ± 11
(No drug)	SHR	6	908.2 ± 16
Nifedipine	WKY	4	627.5 ± 6
	SHR	4	1270.2 ± 7

Increments in ^{3}H-overflow are given in DPM, as sum of increm
55–60 min, 85–90 min, 115–120 min and 145–150 min of superfusion
In nifedipine treated vessels the concentration of the drug in su
$^{\bullet} = p < 0.01$ and $^{*} = p < 0.001$ compared to values in control WKY

TABLE II

ural stimulation induced ^{3}H-overflow from rat tail artery

	DPM/mg wet weight of artery		
-70 min	90–100 min	120–130 min	150–160 min
.7 ± 115.2	410.2 ± 94.3	351.3 ± 68.9	357.5 ± 63.3
.2 ± 163.9	677.0 ± 135.3	665.3 ± 127.1	639.2 ± 114.6
.5 ± 66.4	791.7 ± 15.6●	685.7 ± 51.3●	873.0 ± 39.5●
.2 ± 77.7	1300.0 ± 55.7●	1186.5 ± 75.2●	1158.2 ± 63.4●

ncrements during two subsequent 5 min collections over preceedings 5 min periods, at rfusion, respectively. Mean ± S.E. are shown.
g in superfusate was 10^{-6} M at 90 min, 10^{-5} M at 120 min and 10^{-4} M at 150 min.
WKY or SHR, respectively, with the *t*-test for unpaired samples.

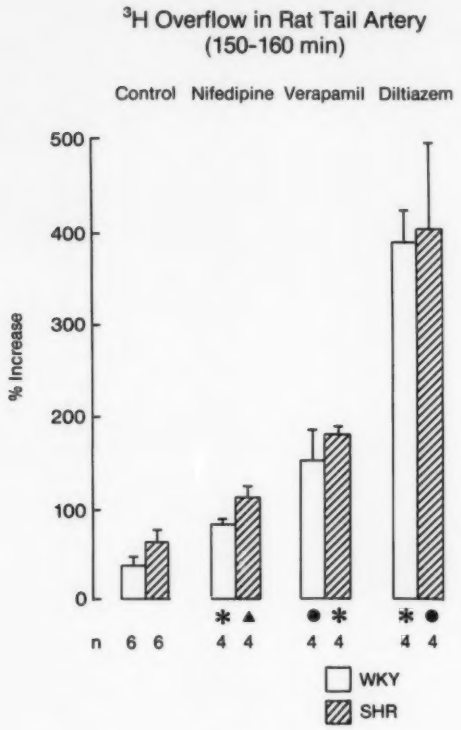

FIG. 1

Increment in ^{3}H-overflow induced by transmural stimulation, expressed as percentage increase in fractional release over values at 145–150 min of superfusion. ▲ = $p < 0.02$, ● = $p < 0.01$ and * = $p < 0.001$ comparing results in untreated arteries with results in vessels perfused with 10^{-4} M nifedipine, verapamil or diltiazem, using the *t*-test for unpaired samples. n = number of animals.

after perfusion with nifedipine. The proportion of NE and its metabolites remained virtually the same after nifedipine. Figure 2B demonstrates that transmural stimulation increased the proportion of norepinephrine and decreased that of dihydroxyphenylglycol both in 8 untreated and 8 nifedipine treated vessels. There was no significant difference between the percentage of norepinephrine or any of its metabolites between control and nifedipine treated arteries neither before nor after stimulation.

If inhibition of Ca^{2+} influx would be the mechanism whereby nifedipine

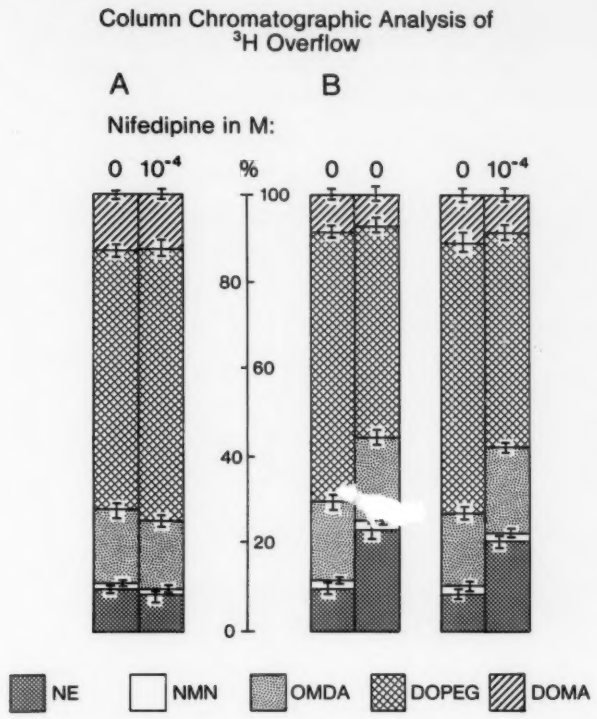

FIG. 2

Percentage of NE and its metabolites in ^{3}H-overflow from rat tail artery analyzed with column chromatography; mean and S.E. are given. Fig. 2A gives results in nonstimulated vessels before and after perfusion with 10^{-4} M nifedipine. Fig. 2B gives results before and after transmural stimulation, at 150–155 min, in untreated and nifedipine treated arteries. Nifedipine concentration in superfusing solution is shown at the top of the figure. NE = norepinephrine, NMN = normetanephrine, OMDA = O-methylated deaminated products, DOPEG = dihydroxyphenylglycol, DOMA = dihydroxymandelic acid.

releases NE from the arteries, one may expect that perfusion of the vessels with O-Ca^{2+} containing solution would prevent the effect of nifedipine. In 2 SHR, at 75 min of superfusion (i.e. after control stimulation) the Krebs solution perfusing the tissues was replaced by an identical solution but without any Ca^{2+}. Transmural stimulation induced increase in ^{3}H-overflow in control SHR was then markedly diminished compared to the effects during superfusion with Krebs solution containing 2.6 mM

Ca^{2+}. In nifedipine (10^{-4} M) treated artery, however, ^{3}H-overflow increased despite superfusion with O-Ca^{2+}; the increment caused by transmural stimulation in presence of nifedipine was greater than after the first stimulation in the absence of the drug (Fig. 3).

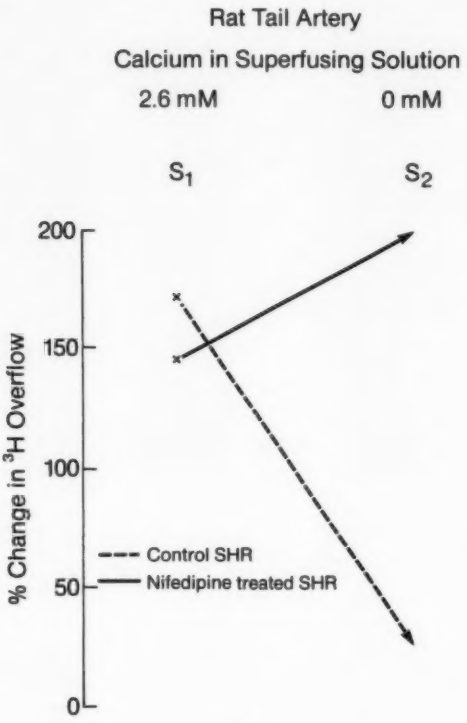

FIG. 3

Effect of transmural stimulation on ^{3}H-overflow during superfusion of the artery with Krebs solution with or without Ca^{2+}. S_1 reflects increase in ^{3}H-overflow during perfusion with Krebs solution containing 2.6 mM Ca^{2+}; % change at 60–80 min over prestimulation value (55–50 min) is shown. S_2 reflects increase during perfusion with solution without Ca^{2+}; change in ^{3}H-overflow at 150–170 min over prestimulation value (145–150 min) is given. ---→ = results in control artery and ⟶ = results in artery perfused with 10^{-4} M nifedipine during second stimulation. S_1 and S_2 = stimulation with 1 Hz, 10 V, 2 msec.

Experiments in Guinea-Pigs

In nonstimulated ^{3}H-NE labelled vas deferens of 6 guinea-pigs, ^{3}H-overflow declined exponentially. The decrease, however, was significantly less during perfusion with 10^{-9} M, 10^{-7} M and 10^{-5} M nifedipine and was replaced by an increase during perfusion with 10^{-4} M nifedipine (Fig. 4). When results were expressed as DPM/mg tissue, regression analysis did indicate a positive correlation between the concentration of the drug and the effect; $p < 0.05$. Increment in ^{3}H-overflow induced by transmural stimulation, however, was not enhanced significantly in other 6 guinea-pigs when perfused with nifedipine.

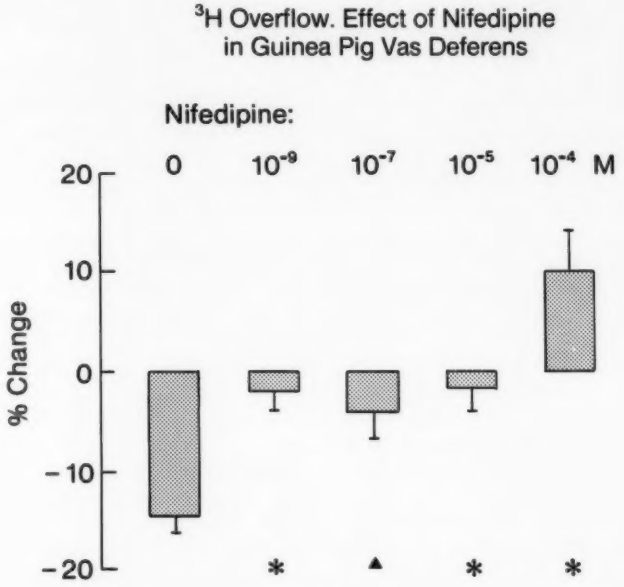

FIG. 4

The effect of nifedipine on ^{3}H-overflow from vas deferens in 6 guinea-pigs. Mean and S.E. of percentage changes for 10 min periods before and after addition of nifedipine ($10^{-9} - 10^{-4}$ M) are shown. ▲ = $p < 0.02$ and * = $p < 0.001$ compared to values in control period before nifedipine was given.

Discussion

Nifedipine increased ^{3}H-release from ^{3}H-NE labelled arteries of SHR and WKY and of guinea-pig vas deferens. Transmural stimulation induced ^{3}H-release was also significantly greater in treated than in untreated vessels of the rat. The effect of nifedipine in these experiments was similar, though less dramatic than that observed with verapamil (Zsotér *et al.*, 1984a) or diltiazem (Zsotér and Wolchinsky, 1985). Since the presentation of our work on verapamil (Zsotér *et al.*, 1984b), other authors reported experiments with results similar to ours; NE release was increased by large concentrations of verapamil (Chaudhry and Vohra, 1984a) and D-600 (Chaudhry and Vohra, 1984b) in rat heart, by diltiazem in rabbit aorta (Karaki *et al.*, 1984) and by verapamil, diltiazem and nicardipine in canine saphenous vein (Takata and Kato, 1984). Thus, several calcium entry blockers can enhance the release of NE from various tissues.

The proportion of NE to its metabolites in ^{3}H-overflow, analyzed with column chromatography, was not altered by nifedipine. This finding is similar to that on diltiazem (Zsotér and Wolchinsky, 1985) and is at variance with that of Chaudhry and Vohra (1984a and b) who found that high concentration of verapamil and D-600 released mainly dihydroxyphenylglycol from rat atrium. Transmural stimulation, as in our previous experiments (Zsotér *et al.*, 1982), increased significantly the proportion of NE and decreased that of dihydroxyphenylglycol in ^{3}H-overflow. This happened to the same extent in nifedipine treated and in untreated vessels. The deamination of NE occurs intracellularly and therefore, the proportion of deaminated metabolites (dihydroxyphenylglycol and dihydroxymandelic acid) in ^{3}H-overflow would be diminished if nifedipine would release ^{3}H from extraneuronal sites. This was not the case and consequently, our results strongly suggest that nifedipine releases NE and its metabolites from sympathetic nerve endings intracellularly.

Ca^{2+} is known to be required for exocytosis of NE. Therefore, the pronounced release of NE after calcium antagonists which block Ca^{2+} influx into the cells, was quite unexpected. We found that superfusion of the arteries with O-Ca^{2+} solution, as expected, almost completedly abolished electrical stimulation induced ^{3}H-release. At the same time NE release by nifedipine and diltiazem (Zsotér and Wolchinsky, 1985) remained virtually unaltered. The results suggest that calcium entry blockers release NE from the tissues by an action other than inhibition of Ca^{2+} influx. We propose that this is an intracellular effect of calcium antagonists on storage sites of NE and its metabolites which is calcium independent like the action of tyramine (Lindmar *et al.*, 1967). The assumption that calcium entry blockers act not only on slow channels but also intracellularly, seems justified in view of some of their other actions (Zsotér and Church, 1983).

In a previous work we found (Zsotér *et al.*, 1982) that transmural stimulation of sympathetic nerves, with 2 or 10 Hz, results in significantly greater release of NE in the SHR than in WKY arteries. Similarly, in the present study stimulation with 1 Hz caused greater ^{3}H-overflow from SHR than WKY both before and after superfusion with nifedipine (Table II). It is important that nifedipine was effective to enhance NE release both in WKY and in SHR.

The greatest effect of nifedipine was observed in concentrations higher than those encountered with its therapeutic use (Henry, 1980). The higher plasma NE levels observed after the clinical use of nifedipine (Lederballe *et al.*, 1979; Kiowski *et al.*, 1983; Kleinbloesem *et al.*, 1984) reflect therefore more likely the activation of baroreceptor reflexes than a direct effect on sympathetic nerve endings. The clinical importance of results described in this paper may be more obvious after the chronic administration of calcium antagonists when they may lead to depletion of NE stores in sympathetic nerves. Our experiments underway should clarify whether this is so. Partial depletion of NE stores after prolonged administration of nifedipine may explain the observations that the first dose of the drug results in greater rise in plasma NE than subsequent doses (Lederballe *et al.*, 1979; Kiowski *et al.*, 1983).

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The Effect of Altering the External Ca^{++} Concentration and TMB-8 on Noradrenaline-Induced Contractions of the Rat Anococcygeus Muscle

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Abstract—The effect of varying external Ca^{++} concentration and 8(-N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) on noradrenaline-induced contractions of the rat anococcygeus muscle was studied. The contractile response to NA was not significantly altered when the external Ca^{++} concentration was reduced by 50 % and 75 %. The contractions were however reduced to approximately 10 % of the control on complete withdrawal of extracellular Ca^{++}. The loss of contractile response to NA in O-Ca^{++} was time-dependent and the rate was not modified by EGTA (0.1 mM). There was a rapid restoration of NA responses on re-introducing Ca^{++}. NA-induced contractions were concentration-dependently antagonized by TMB-8, an intracellular Ca^{++} antagonist. The results are interpreted to suggest that α_1-adrenoceptor activation in the rat anococcygeus muscle releases Ca^{++} from intracellular stores.

Introduction

It is widely accepted that smooth muscle contractile agonists increase the free intracellular cytoplasmic Ca^{++} concentrations to evoke contractions, and that the increase in free cytoplasmic Ca^{++} concentrations could arise from extra or intracellular sources. The mechanism by which noradrenaline (NA) increases the free intracellular Ca^{++} concentrations to evoke contractions of the rat anococcygeus muscle is unknown. Previous studies have shown that NA-induced contractions of the rat anococcygeus muscle were not affected by the organic Ca^{++} entry blockers (McGrath, 1982; Oriowo,

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1982, 1984; Vila *et al.*, 1984). This would suggest either of two possibilities: (1) a transmembrane influx of extracellular Ca^{++} is not involved in NA-induced contractions, or (2) NA activates Ca^{++} channels that are not antagonized by the currently available Ca^{++} entry blockers. In this investigation, we studied the effect of varying external Ca^{++} concentrations and of 8(-N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8), an intracellular Ca^{++} antagonist (Malagodi and Chiou, 1974; Chiou and Malagodi, 1975) on noradrenaline-induced contractions of the rat anococcygeus muscle. An indirect evidence is presented which shows that NA utilizes intracellular Ca^{++} to evoke contraction of the rat anococcygeus muscle.

Methods

Adult male albino rats (180 g–250 g) were killed by a sharp blow to the head and bled through a cut in the neck. The two anococcygeus muscles were isolated and dissected out according to Gillespie (1972). Each anococcygeus muscle was suspended in Tyrode solution (NaCl 137; KCl 2.8, $MgCl_2$ 1.0, NaH_2PO_4 0.3, $CaCl_2$ 1.8, $NaHCO_3$ 11.9 and glucose 5.6 mmol/L) at 37° C contained in a 20.0 ml organ bath. The Tyrode solution was continuously bubbled with air. The resting tension on the tissue was 1.0 g and each preparation was allowed to equilibrate for 60 min (the Tyrode solution was changed at regular intervals). Isotonic contractions (magnification X8) in response to NA were recorded through a frontal writing lever on smoked paper. A tissue drug contact time of 90 sec was allowed and the tissue rested for 5–10 min in between contractions. In some series of experiments, the Ca^{++} content of the Tyrode solution was reduced to 0.9 mM and 0.45 mM while in Ca^{++} Tyrode solution, $CaCl_2$ was simply omitted from the solution.

Effect of TMB-8 on NA-induced contractions of the rat anococcygeus muscle

After obtaining control responses to NA in normal Tyrode solution, appropriate concentrations of TMB-8 were added to the Tyrode solution. The tissues were then allowed to equilibrate with the antagonist for 30 min before repeating the concentration-response curves.

Drugs: The following drugs were used: -(−)-noradrenaline (BDH); 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8, Up John); ethyleneglycol bis(-aminoethylether)-N,N^1 tetraacetic acid (EGTA-Sigma) and diltiazem (Nordic laboratories). EGTA was dissolved in 0.2 M sodium hydroxide while other drugs were dissolved in distilled water.

Results

The effects of reducing Ca^{++} concentrations in the Tyrode solution on NA-induced contractions of the rat anococcygeus muscle is shown in Fig. 1.

There was no significant ($P > 0.05$) reduction in the contractile response to NA when the Ca^{++} concentration was reduced from 1.8 mM to 0.9 mM (1/2) and 0.45 mM (1/4). However, the contractions were reduced to about 10 % or less in 0-Ca^{++} Tyrode solution. In order to assess the rate at which NA-induced contractions were lost in 0-Ca^{++}, control contractions to a submaximal concentration of NA were obtained in normal Tyrode solution and at various time intervals in Ca^{++}-free (0-Ca^{++}) Tyrode solution.

As shown in Fig. 2, there was a gradual decline in NA-induced contractions with time. The contractions stabilized in about 30 min to approximately 10 % with no further reduction. The rate of loss of contractile response was not accelerated by EGTA (0.1 mM) and the residual contractions were still present. Also shown in Fig. 2 is the rate at which responses to NA were restored following the re-introduction of Ca^{++} (1.8 mM final bath concentrations). The results show that between 60–70 %, recovery took place 1 min after re-introducing Ca^{++}. In another series of experiments, concurrent administration of NA and Ca^{++}

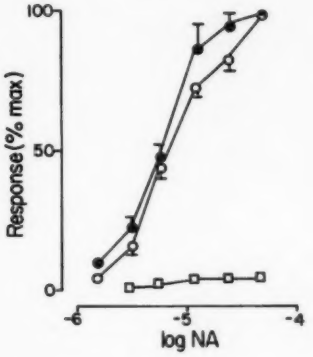

FIG. 1

Effect of reducing external Ca^{++} concentration on noradrenaline-induced contractions of the rat anococcygeus muscle.

● = control

○ = 1/4 Ca^{++} (0.45 mM)

□ = 0-Ca^{++}

Each point is the mean of 4 experiments.

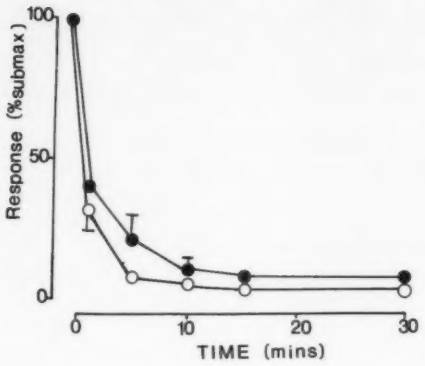

FIG. 2a

Rate of loss of NA-induced contractions of the rat anococcygeus muscle in 0-Ca^{++}.
● = 0-Ca^{++} alone
○ = 0-Ca^{++} + EGTA (0.1 mM)

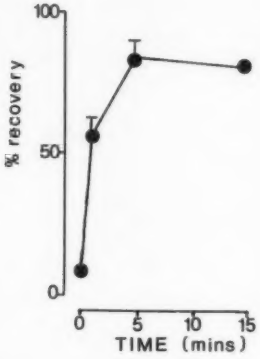

FIG. 2b

The rate of recovery of NA-induced contractions of the rat anococcygeus muscle following Ca^{++} (1.8 mM) reintroduction.

(1.8 mM) did not improve the NA contractions over the contractions in 0-Ca^{++} (Fig. 3) suggesting that an internal store has to be filled before it can be released by NA.

It was therefore interesting to test whether the Ca^{++} entry blocker could prevent the filling of the stores. This was investigated as follows. Contractions to NA were obtained in 0-Ca^{++} until stabilized at about 10 % or

FIG. 3

Typical tracing showing the recovery of the contractile response to NA following reintroduction of Ca^{++} (1.8 mM).
a = NA (▲) 10^{-5} M (in 0-Ca^{++})
b = NA + 1.8 mM administered concurrently
c = NA administered 1 min after Ca^{++} (1.8 mM) added at △

less. Diltiazem (2.5×10^{-6} M) was then added to the bath 30 min before re-introducing Ca^{++}. The contractile response to NA was then obtained 1 min later.

The results (Fig. 4) show that the recovery was still about 70 %, suggesting that diltiazem did not effect the basal influx of Ca^{++} (to fill the stores). Expressing the exponential relationship between the contractile response in 0-Ca^{++} and the concentration of intracellular Ca^{++} at that point in time by the equation

$$\log Ca_s^{++} = \frac{-kt}{2.303} + \log Ca_0$$

where Ca_s is the amount of Ca^{++} in store, t is the time Ca_0, the quantity of Ca^{++} at the beginning of the reaction and k is the rate constant, a plot

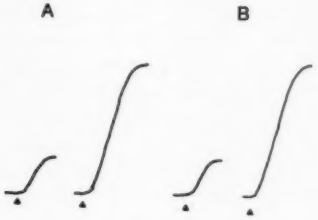

FIG. 4

Typical tracing showing the effect of diltiazem on the rate of recovery of NA-induced contractions following reintroduction of Ca^{++} (1.8 mM).
A = Control response to NA (10^{-5} M in 0-Ca^{++}) before and 1 min after reintroduction of Ca^{++}.
B = Diltiazem (2×10^{-6} M) was added to the bath before the second response to NA was obtained.

of log Ca_s^{++} against t should give a straight line (Hurwitz and Joiner, 1970).

As shown in Fig. 5, a plot of log % response ($=\log Ca_s^{++}$ assuming that the contractile response in 0-Ca^{++} reflects the amount of intracellular

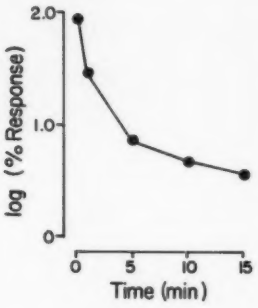

FIG. 5

Contractile effect of NA on the rat anococcygeus muscle in 0-Ca^{++}: Plot of log (% response) against time (min).

Ca^{++}) against t, gave a curve which can be fitted with two tangential lines suggesting that there are possibly two intracellular pools of Ca^{++} which release the sequestered Ca^{++} at different rates.

Effect of TMB-8 on NA-induced contractions of the rat anococcygeus muscle

TMB-8 ($6.25 \times 10^{-6} - 2.5 \times 10^{-5}$ M) had no effect on the basal tone of the anococcygeus muscle. However, these concentrations of TMB-8 (i.e. $6.5 \times 10^{-6} - 2.5 \times 10^{-5}$ M) antagonized NA-induced contractions of the anococcygeus muscle (Fig. 6). There was a suppression of the maximum response. The higher concentration of TMB-8 (2.5×10^{-5} M) was tested in only two preparations because of the limited quantity of the drug available. This concentration of TMB-8 abolished NA-induced contractions of the anococcygeus muscle in these two preparations. The antagonism was irreversible since contractions to NA were not restored even after 1 hr of regular washing with antagonist-free Tyrode solution.

Discussion

Our observation that the contractile response to NA was not significantly altered until the Ca^{++} content of the Tyrode solution was

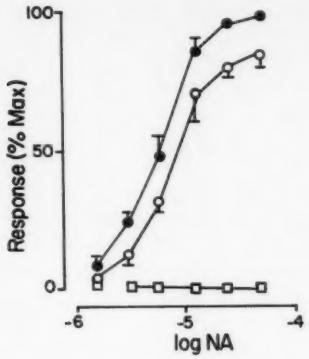

FIG. 6

Effect of TMB-8 on NA-induced contractions of the anococcygeus muscle. TMB-8 was incubated with the tissue for 30 min.

● = control

○ = TMB-8 (6.25×10^{-6} M)

□ = TMB-8 (2.5×10^{-5})

Each point is the mean of 4 experiments except □ where n = 2.

reduced to less than 0.45 mM (i.e. 1/4 of the normal concentration) seems interesting. It does suggest that the amount of extracellular Ca^{++} required for contraction is very low. The obvious consequences of these are: 1) the contractions would be lost in 0-Ca^{++} and 2) the contractions would be resistant to blockade by the Ca^{++} entry blockers apparently because sufficient extracellular Ca^{++} would still permeate the membrane to produce an effect. The logical question could then be: does the influx of this small amount of extracellular Ca^{++} trigger the contractile mechanism directly or does it serve as a trigger to release intracellular Ca^{++} which then activates the contractile mechanism? In an attempt to answer this question, we studied the effect of TMB-8, an intracellular Ca^{++} antagonist (Malagodi and Chiou, 1974; Chiou and Malagodi, 1975) on NA-induced contractions of the anococcygeus muscle. Our results show that TMB-8 concentration-dependently antagonized NA-induced contractions, thus confirming the dependency of NA-induced contractions on intracellular Ca^{++}. Release of intracellular Ca^{++} can take place from the internal surface of the plasma membrane (possibly linked to receptor operated channels) and also from intracellular organelles such as the sarcoplasmic reticulum (Bolton, 1979). It appears that these two sources of Ca^{++} may be involved in NA-induced contractions since the loss of NA-induced contractions in 0-Ca^{++} was characterized by an initial rapid phase followed by a much slower phase. If the influx of extracellular Ca^{++} actually serves as a trigger for the release of intracellular Ca^{++}, then it should be possible to restore the contractions

in 0-Ca^{++} by a concurrent administration of NA and Ca^{++} (1.8 mM). Our results show that this was not the case but that the contractions could be restored to about 60 % if the Ca^{++} was added before NA indicating that the influx of Ca^{++} serves to fill a pool which is subsequently released by NA. This pool can be filled very rapidly even in the presence of diltiazem suggesting that some calcium channels, insensitive to antagonism by diltiazem are involved.

Based on these observations, we are proposing the following scheme: activation of α_1-adrenoceptors in the anococcygeus muscle leads, through receptor operated channels, to a release of membrane bound intracellular Ca^{++} which in turn possibly serves as a trigger mechanism for the release of Ca^{++} from the more tightly bound stores in the organelles. In the normal situation, the Ca^{++} released from the membrane is replaced by Ca^{++} moving in from the extracellular space.

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The Interaction between Nifedipine and Isoprenaline in Isolated Guinea-Pig Tracheal Muscle Precontracted with Methacholine

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Abstract—Our previous studies on the bronchodilator potential of the calcium channel blockers showed that nifedipine antagonised the relaxant effect of isoprenaline in isolated preparations of guinea-pig tracheal muscle which were contracted with cumulative doses of methacholine. For the present study we decided to investigate firstly, whether this interaction would persist in tissue precontracted with methacoline in the presence of isoprenaline and secondly, to investigate the effect of cumulative nifedipine concentrations on any such interaction. Our results show that nifedipine is capable of reversing methacholine-induced contractions with an ID_{50} value of $3{,}5 \times 10^{-8}$ M. However, in tissues precontracted with methacholine in the presence of isoprenaline, nifedipine induces further contraction followed by relaxation, resulting in a biphasic curve. This interaction appears to be a potentially dangerous one and should be investigated further in *in vivo* models.

Introduction

To date, several studies have shown a possible potential benefit of calcium channel blockers in protecting against pharmacological, allergen and exercise-induced bronchoconstriction (Fanta and Drazen, 1983). Collectively, however, it still remains unlikely that the calcium channel blockers will become first-line drugs in the therapy of airways disease. We therefore thought that the efficacy of the calcium channel blockers could be enhanced if they were combined with other bronchodilators. One such study performed in our laboratories (Joubert *et al.*, 1982) showed that

although both nifedipine and isoprenaline on their own blunted methacholine-induced contractions of isolated guinea-pig tracheal muscle, nifedipine antagonized the effect of isoprenaline when these drugs were combined. Further investigation in our laboratories showed that nifedipine inhibits the isoprenaline-induced increase in cyclic AMP in this tissue (Daya and Joubert, 1984). Realizing the potential danger of such an interaction we decided to investigate the effect of nifedipine on isolated tracheal muscle which had already been contracted with methacholine either alone or in the presence of isoprenaline.

Methods

Experiments were performed on the guinea-pig tracheal spiral preparation. The tracheal spirals were prepared as described elsewhere (Constantine, 1965). Each tracheal spiral was suspended in an organ bath (10 ml) containing Krebs solution at 37° C, aerated with 95 % O_2 and 5 % CO_2. A base line tension of 2 g was applied and 20 min allowed for equilibration before the experiments were performed. Each experiment was performed with 6 different preparations on different occasions. The results were expressed as the mean of each experiment ± S.E.M.

Experiment 1

The tracheal spirals were contracted with methacholine at a final bath concentration of 1×10^{-5} M. This was followed by the addition of nifedipine in cumulative concentrations ranging from 1×10^{-11} M to 1×10^{-3} M.

Experiments 2, 3 and 4

For experiment 2, isoprenaline was added to the organ baths at a final bath concentration of 1×10^{-5} M. This was followed by a single dose of methacholine which produced a final bath concentration of 1×10^{-4} M. Once the contraction had been established nifedipine was added as in experiment 1.

For experiment 3, experiment 2 was repeated except that in addition, atropine was added at a final bath concentration of 1×10^{-7} M at the start of the experiment.

For experiment 4, experiment 2 was repeated using Krebs solution containing 5.2 mM calcium instead of the 2.6 mM calcium used in experiments 1, 2 and 3.

Results

The results for experiment 1 are shown in Fig. 1. Nifedipine caused a dose-related relaxation of the tissue in doses ranging from 1×10^{-9} M to 3×10^{-7} M.

The results of experiments 2, 3 and 4 are shown in Fig. 2. Nifedipine induced further contraction of the tissue at concentrations ranging from

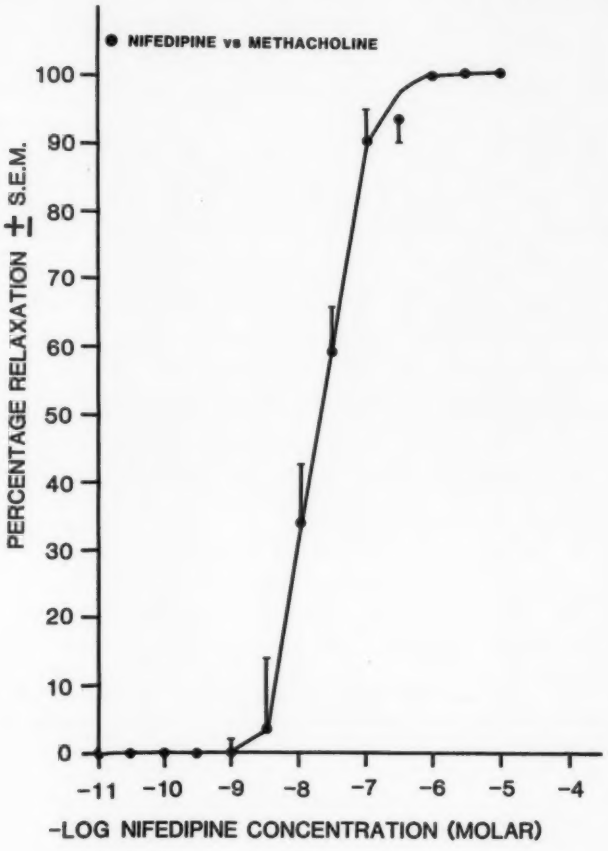

FIG. 1

The effect of cumulative concentrations of nifedipine on methacholine-induced contraction.

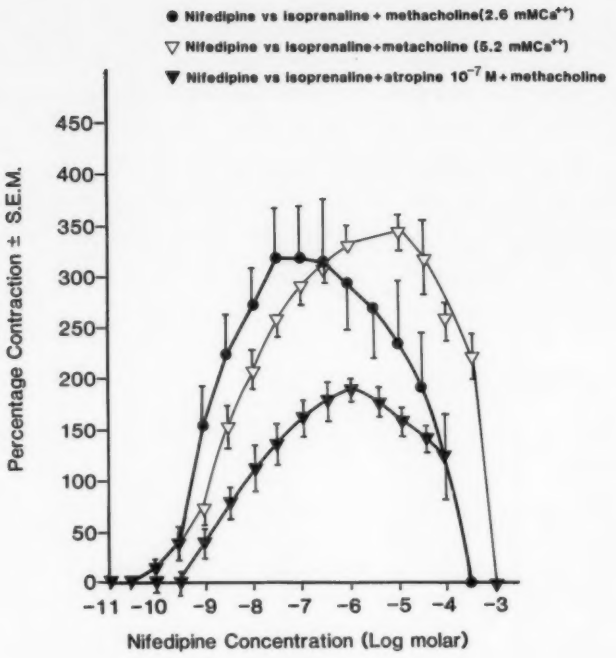

FIG. 2

The effect of cumulative concentrations of nifedipine on tracheal muscle precontracted with methacholine in the presence of isoprenaline ●, isoprenaline plus atropine ▲, and Krebs solution containing 5.2 mM calcium △.

3×10^{-11} M to 1×10^{-7} M and then induced relaxation of the tissue at higher concentrations. The initial methacholine contraction was taken as a 100 % contraction. All further contraction and relaxation was expressed as a percentage of the 100 % initial contraction. The presence of atropine in experiment 3, caused a shift of the ascending limb of the curve to the right. By doubling the calcium concentration in experiment 4, the bell-shaped curve was displaced to the right.

Discussion

Nifedipine appears to act directly on tracheal muscle to cause relaxation of the pharmacologically induced contraction. The ID_{50} value for nifedipine

in this experiment ($3{,}5 \times 10^{-8}$ M) is within the therapeutic range (Patzschke *et al.*, 1975; Williams *et al.*, 1980). The results of experiment 2 show that nifedipine is able to induce further contraction of the tissue in the presence of isoprenaline up to a concentration of 1×10^{-7} M. This could be due to the progressive inhibition by nifedipine of the isoprenaline-induced increase in cyclic AMP (Daya and Joubert, 1984), thus allowing the methacholine to exert further contraction. This view is also supported by the results of experiment 3 which show that the ascending limb of the curve can be antagonized by atropine. By increasing the calcium concentration of the Krebs solution, the relaxation produced by nifedipine occurs at higher nifedipine concentrations as seen in Fig. 2. This implies that the descending limb of the curve is probably due to the calcium channel blocking effect of nifedipine.

This interaction appears to be a potentially dangerous one and should therefore be investigated in *in vivo* models.

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Effect of Nifedipine and Niludipine on Myocardial pH in the Ischemic Canine Heart

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Abstract—The effect of nifedipine and niludipine on ischemic myocardial pH was studied. To make the myocardium ischemic (60 % reduction of coronary flow), the left anterior descending coronary artery (LAD) was partially occluded 30 min before either vehicle (1 % ethanol solution) or drugs were injected intravenously. In the non-occlusion group, nifedipine (10 μg/kg) and niludipine (10 μg/kg) increased coronary flow, decreased blood pressure, and did not change myocardial pH appreciably. In the partial occlusion group, reduction of coronary flow decreased the myocardial pH, increased ST segment, and did not change heart rate and blood pressure. Vehicle was injected 30 min after the occlusion, but did not change any of the parameters. A small dose (1 μg/kg) and a large dose (10 μg/kg) of nifedipine, and niludipine (10 μg/kg) did not exert any beneficial effect on the myocardial pH, coronary flow and the ST segment during coronary occlusion.

Introduction

It has been well established that myocardial pH decreases during ischemia (Gebert *et al.*, 1971; Ichihara *et al.*, 1979; Lange *et al.*, 1983; Watson *et al.*, 1984), and that β-adrenoceptor antagonists attenuate the ischemia-induced decrease in myocardial pH (Ichihara *et al.*, 1979; Abiko and Sakai, 1980; Pieper *et al.*, 1980; Izumi *et al.*, 1982; Nakazawa *et al.*, 1982). Since sotalol, a β-adrenoceptor antagonist, improves the ischemia-induced pH decrease not only in the non-paced normal heart but also in the paced heart (Izumi *et al.*, 1982), the effect of β-adrenoceptor antagonists on the ischemic myocardial pH may not be due to negative chronotropic or membrane stabilizing action of β-adrenoceptor antagonists.

Calcium entry blockers, such as nifedipine, verapamil, and diltiazem can protect the ischemic myocardium probably through their inhibitory action on movements of calcium across the cell membrane (Williams, 1981). We (Ichihara and Abiko, 1982) reported that diltiazem attenuates the ischemia-induced pH decrease in the heart. The mechanism by which diltiazem attenuates the ischemia-induced pH decrease is still unclear, but it seems worthwhile to examine the effect of calcium entry blockers other than diltiazem on the ischemia-induced pH decrease in the heart, because the action of other calcium entry blockers, particularly dihydropyridine derivatives, is not always the same as that of diltiazem (Taira, 1983; Godfraind, 1984; Wellens, 1984).

The present study therefore was undertaken to examine whether nifedipine and niludipine, dihydropyridine derivatives, improve the decrease in myocardial pH that has been decreased by coronary occlusion.

Methods

Ninety-seven mongrel dogs of either sex were used. Dogs were anesthetized with either sodium pentobarbital (30 mg/kg, iv) or morphine-urethane-α-chloralose. When the latter anesthetics were used, dogs were intramuscularly injected with 1.5 mg/kg of morphine hydrochloride, and 30 min after the morphine injection 1 ml/kg of a mixture solution containing urethane (450 mg/ml) and α-chloralose (45 mg/ml) was injected intravenously. Supplemental doses of the mixture solution were given as needed. Artificial respiration with room air was performed by the use of a constant volume respirator (Harvard Apparatus Company, Inc., Millis, MA). A left thoracotomy was performed between the fourth and fifth ribs, and the left ventricular wall was exposed. After a portion of the left anterior descending coronary artery (LAD) was dissected free from the adjacent tissues, an electromagnetic flow probe and an occluder, consisting of a thread, a polyethylene tubing and a fine clip, were placed around the LAD at the level just proximal to the first diagonal branch of the LAD. To measure myocardial pH, a micro glass pH electrode (about 1.0 mm in diameter, MI-410, Microelectrodes, Londonderry, NH) was inserted in the area perfused by the LAD at the depth of 6–8 mm as described earlier (Ichihara *et al.*, 1979). The pH electrode was calibrated with standard pH solutions (pH 6.84 and 7.38) before each experiment, and was calibrated again after the experiment. There was no difference in calibration of the pH electrode between before and after the experiment.

Changes in myocardial pH were recorded continuously with a pen recorder via a pH meter. An epicardial electrocardiogram was taken from the surface of the left ventricular wall by the use of a wire electrode (about 0.05 mm in diameter). One electrode was positioned for each heart in the

area that had been perfused by the occluded coronary artery. Heart rate was counted from the electrocardiogram (standard limb lead II), systolic and diastolic blood pressures were measured in the left carotid artery, and LAD flow was measured by a flow meter (Electromagnetic Flow Meter, Nihon Kohden, Tokyo, Japan) using flow probe placed around the LAD proximal to the occluder. All these parameters were recorded on a polygraph throughout the experiments.

After a stabilization period of 60 min, LAD flow was reduced by the occluder by about 60 % of the original flow (partial occlusion), and maintained constant by adjusting the occluder as required by the method described earlier (Abiko and Sakai, 1980). Briefly, the occluder was connected to the arm of the support by rubber bands. A rubber band was used to prevent rapid changes in LAD flow resulting from the movements of the heart. Coarse adjustment of LAD flow was performed by pulling the thread with the polyethylene tubing and a small clip, while fine adjustment was performed by shifting the arm of the support upward or downward.

After 30 min of partial occlusion, either vehicle solution (0.5 ml/kg), nifedipine or niludipine solution was injected intravenously in the left femoral vein over a period of 30 sec. The LAD was released 60 min after the drug injection. Nifedipine and niludipine were dissolved in ethanol and diluted with saline solution to a final concentration of 20 μg/ml in 1 % ethanol saline solution. Usually 0.5 ml/kg of the drug solution was injected. Accordingly, 10 μg/kg of the drug was injected. As a vehicle, 1 % ethanol-saline solution was employed. The vehicle is expressed as 1 % ethanol in all the figures in the present study.

Each value of myocardial pH measured by the micro glass pH electrode was converted to the hydrogen ion concentration $[H^+]$ according to the following equation.

$$[H^+] = 10^{-pH}$$

Calculation of the mean $\pm$ SE value was made in terms of $[H^+]$, and then the mean value of $[H^+]$ and SE was converted to the pH value again. Statistical analysis for pH changes was made in terms of $[H^+]$ instead of pH value. Results were evaluated by the analysis of Student t-test, and a p value of 0.05 or less was considered significant.

Results

1) *Experiments under Pentobarbital Anesthesia*

Vehicle (1 % Ethanol) Study

In the non-occlusion group (Fig. 1, right), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately

before injection of vehicle were 29.1 ± 3.3 ml/min, 7.54 ± 0.03, 148 ± 13 beats/min, and 142.1 ± 14.2 and 111.4 ± 13.5 mmHg, respectively. An injection of vehicle did not modify any of the parameters determined in the present study.

In the partial occlusion group (Fig. 1, left), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before partial occlusion were 24.2 ± 2.6 ml/min, 7.50 ± 0.02, 150 ± 7 beats/min, and 153.3 ± 5.9 and 122.2 ± 6.0 mmHg, respectively, and those 30 min after partial occlusion (immediately before vehicle injection) were 11.1 ± 2.5 ml/min, 6.82 ± 0.05, 149 ± 7 beats/min, and 154.4 ± 5.7 and 122.8 ± 5.3 mmHg, respectively. Thus myocardial pH decreased by 0.68 in response to partial occlusion that decreased the coronary flow by about 54 %. The ST segment increased by 5.42 mV (compared with the pre-occlusion level) 30 min after partial occlusion. An injection of vehicle did not significantly modify all the parameters studied. Upon release of partial occlusion, coronary flow increased rapidly to the level above the pre-

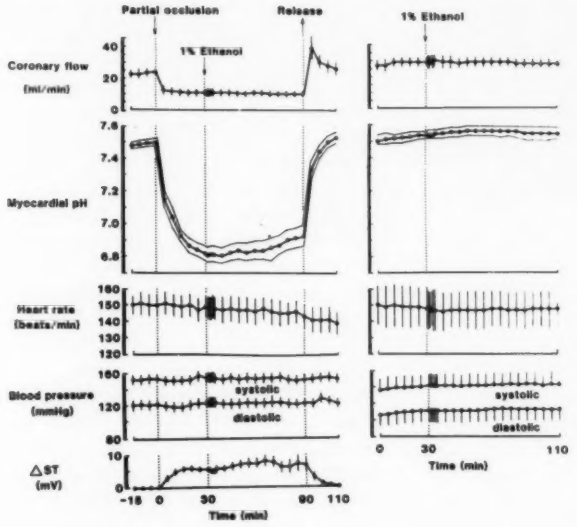

FIG. 1

Effect of vehicle (1 % ethanol) on coronary blood flow, myocardial pH, heart rate, and systolic and diastolic blood pressures in non-occlusion and partial occlusion groups, in which animals were anesthetized with pentobarbital. Changes in ST segment were also recorded in the partial occlusion group. LAD was partially occluded at "0 min" and released at "90 min" in the partial occlusion group. The vehicle was injected intravenously at "30 min". Values are means $\pm$ SEM of 7 (non-occlusion group) and (partial occlusion group) observations.

occlusion level and then returned to the pre-occlusion level, and myocardial pH increased also to the pre-occlusion level. The ST segment that had increased after partial occlusion, decreased to the pre-occlusion level immediately after release of partial occlusion. Thus, vehicle did not affect myocardial pH, heart rate, and blood pressures regardless of the presence or absence of partial occlusion of the LAD.

Nifedipine Study

In the non-occlusion group (Fig. 2, right), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before injection of nifedipine were 20.7 ± 3.5 ml/min, 7.49 ± 0.07, 135 ± 13 beats/min, 135.8 ± 10.7 and 108.3 ± 10.8 mmHg, respectively. An injection of nifedipine (10 μg/kg) increased coronary flow to 33.2 ± 5.5 ml/min (about 60 % increase) 4 min after the drug injection. Both systolic and diastolic blood pressure decreased immediately after the injection of nifedipine. Systolic and diastolic blood pressures 1 min after the nifedipine injection were 97.5 ± 5.7 and 67.5 ± 5.0 mmHg, respectively. The myocardial pH decreased slightly and gradually after the nifedipine injection, but the difference in the pH between before and after the injection was not significant statistically.

In the partial occlusion group, two doses of nifedipine (1 and 10 μg/kg) were tested. In the experiment with 1 μg/kg of nifedipine (Fig. 2, left), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before partial occlusion were 24.3 ± 3.1 ml/min, 7.56 ± 0.02, 160 ± 10 beats/min, and 156.3 ± 6.5 and 124.4 ± 7.2 mmHg, respectively, and those 30 min after partial occlusion were 10.4 ± 2.1 ml/min, 6.83 ± 0.07, 156 ± 11 beats/min, and 148.8 ± 6.9 and 120.0 ± 7.4 mmHg, respectively. The ST segment increases by 4.5 ± 1.4 mV (compared with pre-occlusion level) 30 min after partial occlusion. Thus myocardial pH decreased by 0.73 in response to partial occlusion that decreased the coronary flow by about 58 %. An injection of nifedipine (1 μg/kg) decreased blood pressure, but it did not significantly modify ST segment elevation and myocardial pH. Upon release of partial occlusion, coronary flow increased rapidly to the level above the pre-occlusion flow and then returned to the pre-occlusion level, and myocardial pH also increased to the pre-occlusion level. The ST segment decreased to the pre-occlusion level immediately after release of partial occlusion. Thus nifedipine at a dose of 1 μg/kg did not appreciably modify the myocardial pH and ST segment that had been decreased and increased, respectively, by partial occlusion. In the experiment with 10 μg/kg of nifedipine (Fig. 2, middle), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before partial occlusion were 21.5 ± 2.6 ml/min, 7.64 ± 0.03, 163 ± 5 beats/min, and 156.3 ± 10.3 and

124.4 ± 9.6 mmHg, respectively, and those 30 min after partial occlusion were 9.3 ± 1.8 ml/min, 6.73 ± 0.07, 163 ± 4 beats/min, and 157.4 ± 9.9 and 127.5 ± 9.8 mmHg, respectively. The ST segment increased by 17.40 ± 3.7 mV (compared with the pre-occlusion level) 30 min after partial occlusion. An injection of nifedipine (10 μg/kg) decreased both systolic and diastolic blood pressures, but it did not significantly modify the myocardial pH that had been decreased by partial occlusion; systolic blood pressure decreased maximally to 126.9 ± 9.6 mmHg 1 min after the injection and diastolic blood pressure to 99.4 ± 10.3 mmHg 5 min after the injection. The ST segment increased slightly but significantly after the nifedipine injection. Upon release of partial occlusion, coronary flow and myocardial pH rapidly returned to the pre-occlusion levels. Thus 10 μg/kg of nifedipine decreased blood pressure, but it did not modify the myocardial pH that had been reduced by partial occlusion.

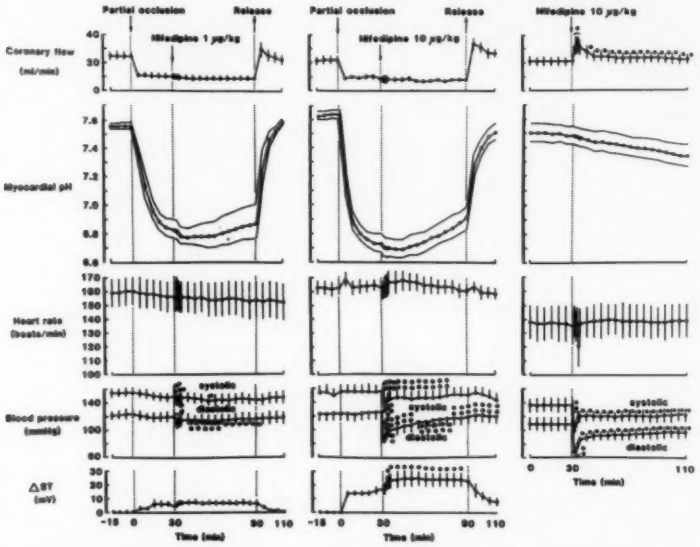

FIG. 2

Effect of nifedipine on coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures in non-occlusion and partial occlusion group, in which animals were anesthetized with pentobarbital. Changes in ST segment were also recorded in the partial occlusion group. LAD was partially occluded at "0 min" and released at "90 min" in the partial occlusion group. Either 1 or 10 μg/kg of nifedipine was injected intravenously at "30 min". Values are means $\pm$ SEM of 6 (non-occlusion group) and 8 (1 and 10 μg/kg of nifedipine in the partial occlusion group) observations.

Niludipine Study

In the non-occlusion group (Fig. 3, right), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before injection of niludipine were 21.5 ± 1.5 ml/min, 7.49 ± 0.07, 128 ± 11 beats/min, and 122.5 ± 11.5 and 92.5 ± 9.2 mmHg, respectively. An injection of niludipine (10 μg/kg) increased coronary flow to 36.0 ± 5.4 ml/min (about 67 % increase) 2 min after the drug injection. Both systolic and diastolic blood pressures decreased immediately after the niludipine injection. Systolic and diastolic blood pressures 3 min after the niludipine injection were 108.3 ± 14.5 and 54.2 ± 7.8 mmHg, respectively. The myocardial pH decreased slightly and temporarily after the niludipine injection, but the decrease in pH was not significant statistically. Heart rate decreased immediately after the niludipine injection significantly.

In the partial occlusion group (Fig. 3, left), coronary flow, myocar-

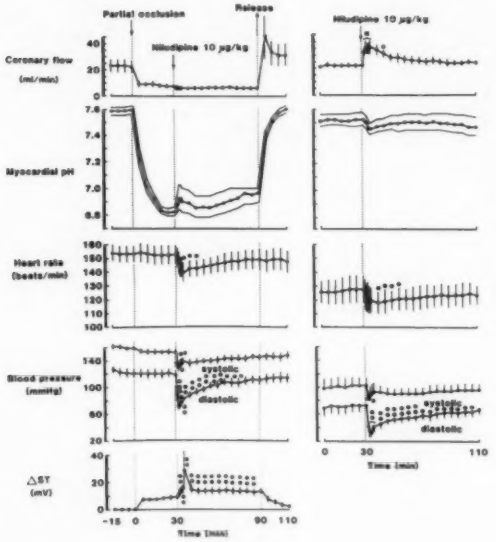

FIG. 3

Effect of nifedipine on coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures in non-occlusion and partial occlusion group, in which animals were anesthetized with pentobarbital. Changes in ST segment were also recorded in the partial occlusion group. LAD was partially occluded at "0 min" and released at "90 min" in the partial occlusion group. Niludipine (10 μg/kg) was injected intravenously at "30 min". Values are means $\pm$ SEM of 6 (non-occlusion group) and 8 (partial occlusion group) observations.

dial pH, heart rate, and systolic and diastolic blood pressures immediately before partial occlusion were 22.6 ± 5.0 ml/min, 7.61 ± 0.04, 153 ± 6 beats/min, and 150.6 ± 3.2 and 113.8 ± 6.5 mmHg, respectively, and those 30 min after partial occlusion were 7.6 ± 1.7 ml/min, 6.83 ± 0.05, 152 ± 7 beats/min, and 144.4 ± 6.5 and 111.9 ± 7.7 mmHg, respectively. Thus myocardial pH decreased by 0.79 in response to partial occlusion that decreased the coronary flow by about 66 %. The ST segment increased by 9.60 ± 2.56 mV (compared with the pre-occlusion level) 30 min after partial occlusion. An injection of niludipine (10 μg/kg) decreased both systolic and diastolic blood pressures, and increased the myocardial pH temporarily, although the increase was significant. The ST segment increased after the injection of niludipine. Heart rate decreased after the niludipine injection significantly. Thus niludipine (10 μg/kg) decreased blood pressure and heart rate, and increased ST segment without a significant change in myocardial pH. Upon release of partial occlusion, coronary flow, myocardial pH, and ST segment returned to the pre-occlusion level.

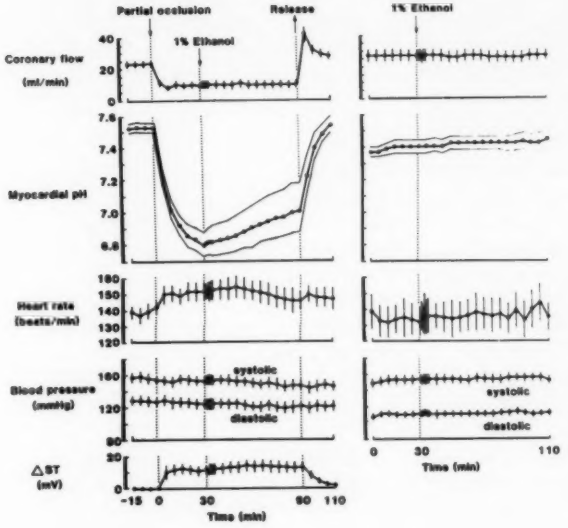

FIG. 4

Effect of vehicle (1 % ethanol) on the parameters in non-occlusion and occlusion groups, in which animals were anesthetized with morphine-urethane-α-chloralose. LAD was partially occluded at "0 min" in the partial occlusion group. The vehicle was injected intravenously at "30 min". Values are means ± SEM of 7 (non-occlusion group) and 8 (partial occlusion group) observations.

2) *Experiments under Morphine-Urethane-α-Chloralose Anesthesia*

Vehicle (1 % Ethanol) Study

In the non-occlusion group (Fig. 4, right), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before injection of vehicle were 26.7 ± 3.7 ml/min, 7.50 ± 0.04, 132 ± 10 beats/min, and 153.6 ± 7.1 and 111.4 ± 4.6 mmHg, respectively. An injection of vehicle did not modify these parameters examined.

In the partial occlusion group (Fig. 4, left), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before partial occlusion were 23.8 ± 2.7 ml/min, 7.53 ± 0.03, 142 ± 4 beats/min, and 154.4 ± 5.9 and 127.5 ± 6.6 mmHg, respectively, and those 30 min after partial occlusion were 9.4 ± 2.4 ml/min, 6.79 ± 0.08, 152 ± 6 beats/min, and 153.8 ± 6.0 and 125.0 ± 5.9 mmHg, respectively. Thus myocardial pH decreased by 0.74 in response to partial occlusion that decreased the coronary flow by about 61 %. The ST segment increased by 11.3 ± 2.75 mV (compared with the pre-occlusion level) 30 min after partial occlusion. An injection of vehicle did not alter the parameters examined. Upon release of partial occlusion, coronary flow and myocardial pH increased and the ST segment decreased, to the pre-occlusion level.

Nifedipine Study

In the non-occlusion group (Fig. 5, right), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before injection of nifedipine were 20.8 ± 6.3 ml/min, 7.47 ± 0.06, 151 ± 5 beats/min, and 176.7 ± 7.9 and 132.5 ± 8.7 mmHg, respectively. An injection of nifedipine (10 μg/kg) increased coronary flow to 36.8 ± 10.7 ml/min (about 77 % increase) 1 min after the injection. Heart rate also increased to 171 ± 12 (about 13 % increase) insignificantly, but systolic and diastolic blood pressures decreased to 163.3 ± 12.6 (about 7.6 % decrease) and 99.2 ± 7.9 (about 25 % decrease) mmHg, 1 min after the nifedipine injection. Myocardial pH, however, did not change after the injection.

In the partial occlusion group (Fig. 5, left), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before partial occlusion were 28.6 ± 5.2 ml/min, 7.53 ± 0.04, 151 ± 12 beats/min, and 173.1 ± 4.8 and 136.3 ± 5.2 mmHg, respectively, and those 30 min after partial occlusion were 12.3 ± 3.2 ml/min, 6.87 ± 0.07, 154 ± 13 beats/min, and 169.0 ± 5.5 and 135.6 ± 4.8 mmHg, respectively. Thus myocardial pH decreased by 0.66 in response to partial occlusion that decreased the coronary flow by about 57 %. The ST segment increased by 10.65 ± 1.87 mV (compared with the pre-occlusion level) 30 min after par-

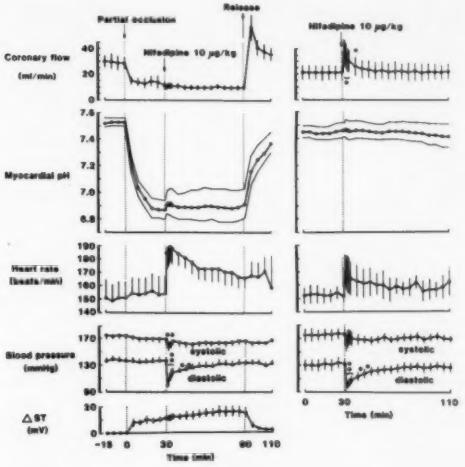

FIG. 5

Effect of nifedipine on each parameter in non-occlusion and partial occlusion group, in which animals were anesthetized with morphine-urethane-α-chloralose. LAD was partially occluded at "0 min" and released at "90 min" in the partial occlusion group. Nifedipine (10 μg/kg) was injected intravenously at "30 min". Values are means ± SEM of 8 observations in each group.

tial occlusion. An injection of nifedipine (10 μg/kg) increased heart rate insignificantly and decreased blood pressure; heart rate increased by 36 beats/min 4 min after the injection, and systolic and diastolic blood pressures decreased by 26.3 and 36.8 mmHg, respectively, 1 min after the injection. However, myocardial pH and ST segment did not change markedly after the nifedipine injection. Upon release of partial occlusion, coronary flow, myocardial pH, and ST segment returned to the pre-occlusion level rapidly.

Niludipine Study

In the non-occlusion group (Fig. 6, right), coronary flow, myocardial pH, heart rate, and systolic and diastolic pressures immediately before injection of niludipine were 27.2 ± 3.5 ml/min, 7.5 ± 0.05, 140 ± 12 beats/min, and 166.7 ± 13.5 and 117.5 ± 11.6 mmHg, respectively. An injection of niludipine (10 μg/kg) increased coronary flow (by about 57 % 2 min after the injection) and heart rate (by about 21 % 2 min after the injection) and decreased systolic and diastolic blood pressures (by about 4.5 and 22.7 %, respectively, 1 min after the injection). Myocardial

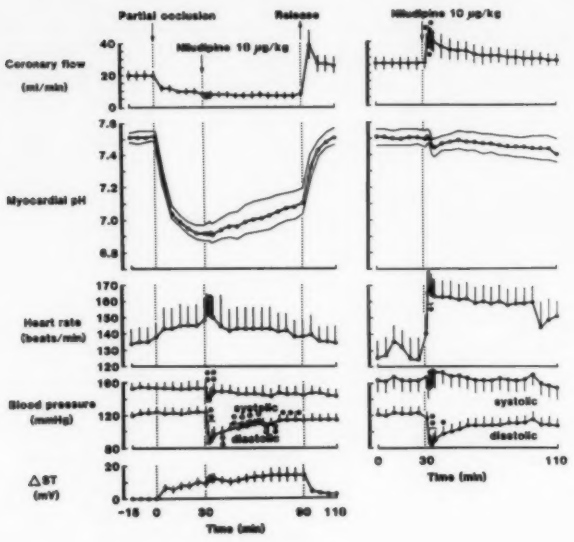

FIG. 6

Effect of niludipine on ech parameters in non-occlusion and partial occlusion groups, in which animals were anesthetized with morphine-urethane-α-chloralose. LAD was partially occluded at "0 min" and released "90 min" in the partial occlusion group. Niludipine (10 μg/kg) was injected intravenously at "30 min". Values are means ± SEM of 6 (non-occlusion group) and 8 (partial occlusion group) observations.

pH slightly and temporarily decreased immediately after the niludipine injection. The change in myocardial pH, however, was not significant statistically.

In the partial occlusion group (Fig. 6, left), coronary flow, myocardial pH, heart rate, and systolic and diastolic blood pressures immediately before partial occlusion were 20.4 ± 2.7 ml/min, 7.52 ± 0.03, 138 ± 9 beats/min, and 154.4 ± 6.5 and 123.8 ± 5.9 mmHg, respectively, and those 30 min after partial occlusion were 8.1 ± 1.8 ml/min, 6.92 ± 0.05, 148 ± 14 beats/min, and 152.5 ± 7.6 and 123.8 ± 7.6 mmHg, respectively. Thus myocardial pH decreased by 0.6 in response to partial occlusion that reduced the coronary flow by about 60 %. The ST segment increased by 8.70 ± 1.48 mV (compared with the pre-occlusion level) 30 min after partial occlusion. An injection of niludipine (10 μg/kg) did not significantly modify the myocardial pH, ST segment elevation, and heart rate, but it decreased systolic and diastolic blood pressures significantly (by about 8.9 and 21 %, respectively, 1 min after the injection). Upon release of partial occlusion,

coronary flow, myocardial pH, and ST segment returned to the pre-occlusion level rapidly.

Discussion

It has been demonstrated that nifedipine prevents myocardial contracture during ischemia (Henry *et al.*, 1977), and reduces ischemic injury induced by coronary occlusion (Henry *et al.*, 1978). It has also been shown that myocardial cells undergo ischemic damage including mitochondrial and nuclear alterations after coronary occlusion (Jennings *et al.*, 1969; Schaper *et al.*, 1979), and that nifedipine partially prevents these morphological changes induced by ischemia (Sashida *et al.*, 1982). Because calcium accumulates in the mitochondria after coronary occlusion (Shen and Jennings, 1972; Buja *et al.*, 1976; Ashraf *et al.*, 1976; Ashraf and Bloor, 1978), the protective action of nifedipine, a calcium entry blocker, on the ischemic myocardial damage could be due to its inhibitory action on the calcium influx (Fleckenstein, 1971).

Coronary artery occlusion produces myocardial pH decrease (Gebert *et al.*, 1971; Ichihara *et al.*, 1979). A reduction of myocardial pH may have the advantage to the myocardium in an early period of ischemia, because acidosis decreases myocardial contractility, resulting in decreasing energy demand of the heart (Katz, 1973). If this acidic state is prolonged, however, irreversible myocardial cell damage will occur. We (Ichihara and Abiko, 1982) reported that diltiazem, another calcium entry blocker, attenuates myocardial pH decrease induced by coronary occlusion. The results of the present study, however, indicated that nifedipine and niludipine did not attenuate pH decrease induced by coronary occlusion in dogs anesthetized with either pentobarbital or morphine-urethane-α-chloralose. Because both nifedipine and niludipine increased coronary flow in the non-occlusion groups, the dose of these drugs tested was considered enough to produce the pharmacological effect in terms of coronary vasodilatation. The reason why nifedipine and niludipine did not restore myocardial pH that had been reduced by partial occlusion of the coronary artery is unknown, but it is possible to assume that the dose of these drugs was not sufficient to attenuate the myocardial pH decrease, and that these drugs were given after the occlusion. The dose of nifedipine that is effective in protecting morphological changes induced by coronary occlusion is 100 μg/kg (Sashida *et al.*, 1982). They (Sashida *et al.*, 1982) have also reported that neither nifedipine nor niludipine given after coronary occlusion exerts any effect on the morphological changes induced by ischemia, whereas these drugs given before and during ischemia inhibit the ischemic myocardial cell damage. Pretreatment with nifedipine and niludipine could be important to exert their beneficial effects on the myocardium. According to Selwyn *et al.*

(1979), treatment with nifedipine at the dose of 1 μg/kg improves the regional perfusion in ischemic myocardium, while 13 μg/kg nifedipine results in an extension of infarct size. Kloner and Braunwald (1980) have mentioned in their paper that measurement of infarct size using the triphenyl tetrazolium chloride technique developed in their laboratory is unable to show that nifedipine in various doses has an effect on infarct size.

Recently, the difference in pharmacological properties between dihydropyridine derivatives, such as nifedipine and niludipine, and other calcium entry blockers, such as diltiazem and verapamil, is pointed out; the former affects the vascular smooth muscle more selectively than the cardiac muscle, whereas the latter affects both vascular smooth muscle and cardiac muscle (Taira, 1983). Peters (1983) has demonstrated that there are at least three mechanisms by which the calcium gradient at the level of the cellular membrane is maintained: 1) membrane stabilization, 2) blockade of specific calcium channels, and 3) improvement of the calcium extrusion pump. Nifedipine and niludipine may act on calcium channels specifically, while diltiazem may have an additional effect on the stability of the cell membrane and the calcium extrusion pump. This may be the reason why the response of the myocardial pH during ischemia to nifedipine and niludipine is different from that to diltiazem.

Pentobarbital is usually used as an anesthetic agent in our laboratory. In dogs under pentobarbital anesthesia, we were unable to observe any effect of nifedipine and niludipine on the ischemic myocardial pH. Because neural reflex activity under α-chloralose anesthesia is better retained, and because changes in ventricular performance after α-chloralose anesthesia are less than those after pentobarbital anesthesia (Citters *et al.*, 1964), we repeated the same experiments under morphine-urethane-α-chloralose anesthesia. Difference in anesthesia did not modify the results with myocardial pH and blood pressure. Blood pressure was decreased by the nifedipine or niludipine injection in either non-occlusion groups in dogs anesthetized with pentobarbital or morphine-urethane-α-chloralose. Heart rate response to these drugs, however, was modified by difference in anesthesia (Figs. 2, 3, 5, and 6).

A micro glass pH electrode may not be an accurate tool for assessing the myocardial pH compared with ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR) (Pieper *et al.*, 1980). However, it is rather difficult to apply the ^{31}P NMR method to *in vivo* study at the present, although Grove *et al.* (1980) have analyzed rat heart *in vivo* using the NMR receiver coil. Numerous reports on ischemic myocardial pH change and on effects of drugs on the myocardial pH change have been piled up. The results obtained in experiments using a micro glass pH electrode (Ichihara *et al.*, 1979; Walters *et al.*, 1979; Abiko and Sakai, 1980; Lange *et al.*, 1983) or using fibroptic pH probe (Watson *et al.*, 1984) are not essentially different from those using ^{31}P NMR method (Hollis *et al.*, 1978; Garlick *et al.*, 1979;

Grove *et al.*, 1980; Pieper *et al.*, 1980; Nakazawa *et al.*, 1982; Brooks and Willis, 1983).

From the foregoing results, it is concluded that nifedipine (1 or 10 μg/kg) and niludipine (10 μg/kg) do not modify myocardial pH that has been decreased by partial occlusion of the coronary artery in dogs anesthetized with either pentobarbital or morphine-urethane-α-chloralose.

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Inhibition of Quinidine-Propranolol Elevation of the Ventricular Fibrillation Threshold by Bilateral Stellectomy + Vagotomy in the Anesthetized Dog ([1])

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Abstract—The antiarrhythmic potentiation that occurs with the coadministration of quinidine-propranolol has been attributed to propranolol-induced cardiac beta adrenergic receptor blockade. That this may not be the sole mechanism, however, is suggested by the finding that sotalol, pronethalol and practolol fail to potentiate quinidine in some antiarrhythmic tests. Thus, in the present experiments, the influence of bilateral stellectomy + vagotomy on quinidine-propranolol antiarrhythmic activity was studied in the anesthetized dog. Antiarrhythmic activity was determined as elevation of the electrical ventricular fibrillation threshold (VFT) when the left ventricle was stimulated directly with 60 Hz, 6 msec and varying mA for 2 sec. The increase in VFT produced by quinidine-propranolol (5 + 0.2 mg/kg, i.v.) was significantly reduced when the heart was subsequently denervated by bilateral stellectomy + vagotomy. The cardiac denervation did not significantly reduce the elevation of VFT that had been produced by prior administration of propranolol 0.4 mg/kg or sotalol. In other groups with *prior* bilateral stellectomy + vagotomy, the separate administration of propranolol 0.4 mg/kg or quinidine 5 mg/kg failed to significantly elevate the VFT although the VFT was increased by propranolol 0.8 mg/kg. The results indicate that the action of quinidine-propranolol to elevate the VFT may be dependent in part upon an intact nerve supply to the heart.

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Introduction

Propranolol and some other beta adrenergic blocking agents elevate the ventricular fibrillation threshold (VFT) and the decreased ventricular vulnerability has been suggested as a possible mechanism by which beta blocking drugs reduce sudden cardiac death (Anderson *et al.*, 1983). Propranolol has also been shown to potentiate the antiarrhythmic activity of quinidine in various experimental animal tests (Lawson, 1968; Lawson and Wojciechowski, 1974; Lawson and Wojciechowski, 1977) and this combination is also effective in treating arrhythmias in man (Stern, 1966; Levi and Proto, 1972; Reynold and Vanderark, 1967). The mechanism of quinidine potentiation by propranolol has been attributed to the cardiac beta adrenergic receptor blocking action of propranolol (Iansmith *et al.*, 1981). It has been found, however, that the production of an approximately equivalent degree of cardiac beta receptor blockade by sotalol in the mouse and dog (Lawson and Wojciechowski, 1981) and by practolol and pronethalol in the mouse (Lawson, 1984) did not potentiate quinidine. Furthermore, a dose of d-propranolol so small as to be essentially devoid of cardiac beta receptor blocking activity potentiated quinidine in the mouse (Lawson, 1984). Thus, some other action of propranolol may contribute to the potentiation. Since propranolol is known to decrease sympathetic outflow (Eliash and Weinstock, 1972), it was the purpose of the present investigation to determine the effect of bilateral stellectomy + vagotomy, which interrupt a major portion of cardiac sympathetic outflow, on quinidine-propranolol antiarrhythmic activity.

Methods

Mongrel dogs of either sex were anesthetized with i.v. *alpha*-chloralose (60 mg/kg) and urethane (185 mg/kg) plus pentobarbital sodium (3 to 6 mg/kg). An electric heating pad was used to maintain body temperature (rectal) at 37–39° C. Blood pressure was measured from a cannulated common carotid artery using a Statham P23AC transducer and recorded on a Grass polygraph. Heart rate was counted from the blood pressure tracing. The animals were placed on artificial positive pressure respiration, the thorax opened via a midline incision and the heart exposed for electrical fibrillation by suspending it in a pericardial cradle. The surface of the heart was kept moistened with warm 0.9 % NaCl and the thoracic opening covered with Saran wrap. Measurement of the VFT was carried out as previously described (Lawson and Wojciechowski, 1977). Briefly, the heart was fibrillated by applying square wave stimuli consisting of 60 Hz, 6 msec

duration and varying mA directly to the surface of the left ventricle (apex) for 2 sec. The VFT was determined by measuring the minimal or threshold current required to fibrillate the heart, whereafter, defibrillation was accomplished immediately by electrical countershock. The VFT was found by applying stimuli of increasing magnitude, with each stimulus increased from the preceding one by 0.05 mA through 0.3 mA, and thereafter by 0.1 mA. The VFT was determined just preceding and following i.v. administration of the antiarrhythmic drugs or surgical interruption of the neural connections to the heart. In most instances, the VFT was calculated as the mean of two VFT measurements. Due to the length of time required to measure the threshold, however, one exception was when determining the duration of the effect of quinidine + propranolol on the VFT where only single measurements were used. Another exception was when determining the effect of denervation on the VFT that had been previously elevated by the antiarrhythmic drugs. In this case, the difference between the last VFT measured just preceding denervation and the first VFT measured just following denervation was used. An increase in VFT, i.e. a decrease in ventricular vulnerability, was considered a measure of antiarrhythmic activity. Ventricular fibrillation was produced at intervals of 10 min or longer; at these intervals, acid-base balance can be maintained up to 5 hr (Spear *et al.*, 1972; Battle *et al.*, 1974). Open-chest dogs which show progressive metabolic acidosis also show a progressive decrease in VFT (Spear *et al.*, 1972). With the presently described method, the VFT is not changed significantly through 8 consecutive fibrillation-defibrillation procedures, which require approximately 2 hr to complete (Lawson and Wojciechowski, 1974). Change in heart rate does not have a consistent effect on the VFT in the non-ischemic heart (Kent *et al.*, 1973) until a change in rate of 100 to 150 b/min occurs (James *et al.*, 1977). Thus, in the present study, ventricular rate was not controlled because preliminary experiments indicated that heart rate changes of that magnitude would not be encountered.

Partial cardiac denervation was produced in some animals as follows. The right and left stellate ganglia were identified and exposed by careful dissection. The main body of each ganglion was then extirpated by grasping and elevating it with a hemostat, while sectioning all pre- and postganglionic fibers by cautery using a 1 mm pencil-type soldering iron. Both vagosympathetic trunks were sectioned in the midcervical region. The combined stellectomy + vagotomy is referred to below as cardiac denervation.

All antiarrhythmic drugs were solubilized in 0.9 % NaCl and administered via a cannulated femoral vein, with quinidine and quinidine-propranolol infused slowly to minimize the hypotensive effect. When quinidine and propranolol were coadministered, quinidine SO_4 (5 mg/kg) in a volume of 16 ml was combined with propranolol HCl (0.2 mg/kg) in

4 ml and infused from a single syringe using a Harvard model 600–900 VDC infusion pump.

The significance of the effects of the drugs and of surgical interruption of the neural connections to the heart on the VFT and heart rate were determined using paired analysis. Student's *t*-test was used and a probability level of $<.05$ considered significant. All data are presented as the mean $\pm$ S.E.

Results

Quinidine-propranolol ($5 + 0.2$ mg/kg) was administered during 34 ± 3 min to a control group of 7 neurally intact dogs and the duration of the elevation of VFT produced was determined. Four postdrug measurements were made. The first at 9 ± 1 min after administering the antiarrhythmic drugs and the 2nd, 3rd and 4th were made at 22 ± 2, 37 ± 2 and 54 ± 2 min following the first measurement. The predrug control VFT in this group was 0.19 ± 0.014, which was increased to 0.44 ± 0.030 mA ($P < .001$) at 9 min following the antiarrhythmic drugs. The VFT decreased with time but the values of 0.34 ± 0.079, 0.31 ± 0.050 and 0.31 ± 0.048 mA that were determined at 21, 37 and 54 min, respectively, were not significantly reduced from the value obtained at 9 min ($P > .05$).

An additional group of 7 neurally intact dogs were administered quinidine-propranolol ($5 + 0.2$ mg/kg) during 37 ± 2 min. The control VFT of 0.20 ± 0.030 was increased to 0.68 ± 0.141 mA ($P < .01$) as determined at 11 ± 0.9 min after administering the drugs (Table I). The heart was then immediately denervated and the VFT measurement repeated, as in the above control group, at 21 ± 2 min, at which time the VFT was reduced to 0.28 ± 0.056 mA ($P < .05$). There was no significant difference in the respective blood pressure and heart rate values that existed when the VFT was measured just before and just after cardiac denervation, which suggested that the denervation procedure had contributed importantly to the resulting fall in VFT.

Propranolol (0.4 mg/kg) was administered to a group of 6 neurally intact dogs and the VFT was elevated from the control value of 0.17 ± 0.026 to 0.32 ± 0.053 mA (Table 1; $P < .01$). Thereafter, cardiac denervation failed to have a significant effect on ventricular vulnerability as the mean VFT remained at 0.36 ± 0.076. The elapsed time between these two VFT measurements was 25 ± 0.8 min. Heart rate was reduced from 141 ± 8 to 130 ± 10 b/min ($P < .05$). Such a small change in heart rate does not have a significant effect on VFT whether measured in the neurally intact animal (Kent *et al.*, 1973; James *et al.*, 1977) or in the dog with bilateral stellectomy + vagotomy (Han, 1966).

Sotalol HCl was administered to another group of 6 neurally intact dogs

TA

The Effect of Bilateral Stellectomy and Vagotomy
Threshold Produced by Quinidine + Propranolol,

Drug Treatment	Dose mg/kg, i.v.	N ([3])
Quinidine + Propranolol	5.0 + 0.2	7
Propranolol	0.4	6
Sotalol	5.1 ([6])	6

([1]) Bilateral stellectomy + vagotomy performed *after* fibrillation thresh
([2]) Milliamps (mean ± S.E.).
([3]) Number of animals.
([4]) Significance was analyzed by pairing where the difference in VFT j
animal.
([5]) Significance was analyzed by pairing where, after drug administratio
was calculated for each animal.
([6]) Cumulative dose was 5.1 ± 1.16 mg/kg, i.v.
* $P < .05$, *** $P < .01$.

TABLE I

tomy on the Increase in the Electrical Ventricular Fibrillation
nolol, Propranolol, and Sotalol in the Anesthetized Dog (1)

Fibrillation Threshold (mA) (2)		
Control Before Drug	After Drug (4)	After Stellectomy + Vagotomy (5)
0.20 ± 0.030	0.68 ± 0.141***	0.28 ± 0.056*
0.17 ± 0.026	0.32 ± 0.053***	0.35 ± 0.076
0.19 ± 0.064	0.43 ± 0.096*	0.26 ± 0.044

threshold elevated by drug treatment.

VFT just before drug versus just after drug administration was calculated for each

istration, the difference in VFT just before denervation versus just after denervation

L

0

S

R

6

I

that had a control VFT of 0.19 ± 0.064 mA (Table I). This beta blocker was given in 2 to 3 divided doses and the VFT measured after each dose. The mean total cumulative dose was 5.1 ± 1.16 mg/kg, i.v. After the total dose had been administered, the VFT was increased to 0.43 ± 0.096 mA ($P < .05$). Thereafter, cardiac denervation failed to have a significant effect on ventricular vulnerability as a mean VFT of 0.26 ± 0.044 was measured ($P > .05$). The elapsed time between these two VFT measurements was 23 ± 2 min. Heart rate was not significantly changed.

Three additional groups of 7 animals each were prepared and they differed from the above animals in that cardiac denervation was produced *before* administering the antiarrhythmic drugs. One group was administered propranolol 0.4 mg/kg and Table II shows that the control VFT was elevated from 0.16 ± 0.021 to only 0.21 ± 0.039 mA ($P > .05$). This

TABLE II

The Effect of Prior Stellectomy and Vagotomy on the Electrical Ventricular Fibrillation Threshold as Produced by Propranolol and Quinidine in the Anesthetized Dog (1)

Drug Treatment	Dose mg/kg, i.v.	N (3)	Fibrillation Threshold (mA) (2)	
			Before Drug	After Drug (4)
Propranolol	0.4	7	0.16 ± 0.021	0.21 ± 0.039
Propranolol	0.8	7	0.17 ± 0.023	0.45 ± 0.079**
Quinidine	5.0	7	0.21 ± 0.040	0.30 ± 0.075

(1) Bilateral stellectomy + vagotomy performed *before* drug administration.
(2) Milliamps (mean ± S.E.).
(3) Number of animals.
(4) Significance was analyzed by pairing where the difference in VFT just before drug versus just after drug administration was calculated for each animal.
** $P < .025$.

is in contrast to the results obtained in the neurally intact group described above where 0.4 mg/kg of propranolol elevated the VFT from 0.17 to 0.32 mA ($P < .01$). The second group was administered propranolol 0.8 mg/kg and the control VFT of 0.17 ± 0.023 was increased to 0.45 ± 0.079 mA (Table II, $P < .025$). Heart rate was not significantly changed. The third group of denervated animals was administered quinidine 5 mg/kg and the control VFT of 0.21 ± 0.040 was increased to only 0.30 ± 0.075 (Table II, $P > .05$). Heart rate was not significantly changed.

Discussion

Although quinidine is a clinically useful antiarrhythmic drug, it has only a slight effect to increase the VFT (Bacaner, 1968; Lawson and Wojciechowski, 1974). Since quinidine may increase cardiac adrenergic activity by an action mediated via sympathetic pathways (Roberts *et al.*, 1962) and since an increase in cardiac sympathetic tone lowers the VFT (Han *et al.*, 1964), it was of interest in the present study to determine whether quinidine might produce a more substantial increase in VFT after cardiac sympathetic tone had been reduced by prior stellectomy + vagotomy. A dose of 5 mg/kg did not significantly increase the VFT.

The results obtained when quinidine was coadministered with propranolol to the neurally intact dog suggest that subsequent interruption of the nerve supply to the heart may have interfered with the drug-induced elevation of the VFT. Other experiments would be necessary to determine whether the interference is due principally to interruption of neural pathways associated with the stellate ganglia, the vagosympathetic nerve trunks, or perhaps to both. An elevated sympathetic tone to the heart would be expected to have been present in these open-chest animals and since an increase in sympathetic tone results in a fall in VFT, bilateral stellectomy together with interruption of sympathetic efferent fibers coursing in the vagus nerve might be expected to have resulted in an increase in VFT, not a decrease as was observed under the conditions of the present experiments. Since interruption of cardiac afferent sympathetic nerves might similarly be expected to increase the VFT (Malliani *et al.*, 1973), the effect of bilateral stellectomy + vagotomy to reduce quinidine-propranolol elevation of the VFT may be due relatively more to interruption of vagal nerve activity.

Vagal stimulation has been reported to markedly increase the VFT and these investigators believed that the elevation was mediated directly by cholinergic fibers supplying the ventricular conduction system (Kent *et al.*, 1974). Others, however, have contended that vagal stimulation may have little or no effect on ventricular vulnerability and if it does increase the VFT, the effect is not direct; rather, it is due to suppression of cardiac sympathetic activity that results indirectly from an increase in vagal afferent or cholinergic efferent nerve tone (Kolman *et al.*, 1975; Rabinowitz *et al.*, 1976; Brooks *et al.*, 1978).

Propranolol elevates the VFT in the neurally intact dog as well as in the dog with bilateral stellectomy + vagotomy (Lawson and Wojciechowski, 1974; Euler, 1980; Anderson *et al.*, 1983) and the magnitude of the elevation was greater when the VFT was measured by the trains of stimuli as compared with the single stimulus technique (Euler, 1980).

Norepinephrine may be released locally at the site of applying the stimulus and may have a profound effect on the measured level of VFT (Kolman, 1975; Euler, 1980). Thus, it might be supposed that the mechanism of propranolol to increase the VFT could include inhibition of the release of norepinephrine and/or of the action of the released catecholamine on beta receptors.

The present experiments do not explain why bilateral stellectomy + vagotomy failed to lower the VFT that had been elevated by propranolol and sotalol when denervation did appear to reduce the VFT that had been elevated by quinidine-propanolol. The degree of cardiac beta receptor blockade produced by these three drug treatments would be expected to be of similar magnitude (Lawson and Wojciechowski, 1981). Neither do the present experiments explain why prior cardiac denervation appeared to inhibit the development of a significant increase in VFT to 0.4 mg/kg of propranolol but not to 0.8 mg/kg. The smaller dose would be expected to produce cardiac beta receptor blockade and a dose larger than 0.8 mg/kg is generally thought necessary to produce a direct quinidine-like effect in the dog. If the reduction in quinidine-propranolol elevation of the VFT by the cardiac denervation was due to loss of vagal inhibition of cardiac norepinephrine release, it would seem that the denervation should have had a similar effect in animals administered only propranolol or sotalol.

If the mechanism of quinidine-propranolol elevation of the VFT should be found to include an action mediated via the vagus, it would not negate an important contributing effect of beta receptor antagonism nor of the possible contribution of a more direct action of the drug combination on cardiac fibers to prolong the effective refractory period (Korte and Nash, 1976).

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Interaction between Adenosine and Antiarrhythmic Agents in Atrial Myocardium of Guinea-Pig

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Abstract—The effect of quinidine (class 1 agent) and verapamil (class 4 agent) on the adenosine-induced changes in the electrical and mechanical activity of the left atrial myocardium of guinea-pig was studied. Adenosine alone shortened the action potential duration, slightly hyperpolarized the membrane and increased the $\dot{V}_{max}$ as well as induced a negative inotropic effect. Quinidine, a membrane-stabilizing drug, prolonged the action potential duration, decreased the $\dot{V}_{max}$, and increased the contractile force, i.e. diminished the electrical and mechanical changes in the atria treated with adenosine. These effects became more pronounced in the presence of aminophylline, a P_1-purinoceptor blocker. In contrast, verapamil, a Ca-channel blocker, enhanced the adenosine-induced shortening in the early repolarization phase of the action potential, and the negative inotropic effect. This action of verapamil could be attenuated with aminophylline. The results suggest that adenosine can influence the effect of antiarrhythmics compounds and vice versa, and that the action of adenosine is possibly mediated via interaction with an extracellular P_1-purinoceptor.

Introduction

Berne (1963) was the first to describe that adenosine, a physiological metabolite released from cardiac cells mainly during myocardial hypoxia, plays an important role in the regulation of the coronary blood flow. Since

then, numerous efforts have been made in conforming and developing this attractive theory. This purine nucleoside shortens atrial muscle transmembrane action potentials, exerts a negative inotropic effect (Drury and Szent-Györgyi, 1929; De Gubareff and Sleator, 1965) and reduces the slow action potentials mediated mainly by slow Ca-channels (Schrader *et al.*, 1975; Belardinelli *et al.*, 1979; Mészáros *et al.*, 1984). These authors also suggested that the adenosine-induced negative inotropic effect might be due to a decrease of the Ca-influx during excitation in guinea-pig atrial heart muscle, and that the effect of adenosine is mediated by an extracellular P_1-purinoceptor (see also Burnstock, 1976; Schrader *et al.*, 1977). Recently, it has been shown that adenosine increases potassium conductance (Goto *et al.*, 1977; Nawrath and Jochem, 1982; Belardinelli and Isenberg, 1983; Hutter and Rankin, 1984; Mészáros *et al.*, 1984).

The purpose of the present study was to determine whether there is an antagonism or interaction between adenosine and two well-known and widely used antiarrhythmic drugs, quinidine (class 1 agent) and verapamil (class 4 agent).

Methods

The experiments were carried out on isolated, electrically driven left atrial preparations of guinea-pig hearts. Animals of either sex, with a body weight of 250–350 g were killed by a sharp blow on the skull. Their hearts were quickly removed and placed in oxygenated Krebs solution at room temperature. After dissection, the left atria were mounted horizontally in a jacketed 5 ml organ chamber. All preparations were allowed to equilibrate for 30 min in Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.0, $NaHCO_3$ 24.9, glucose 11.5. The solution was aerated with a gas mixture of 95 % O_2 and 5 % CO_2, the pH was 7.4, and the temperature was maintained at 37° C.

Isometric contractions of isolated left atrial preparations of guinea-pigs were measured by means of an isometric force transducer. The preparations were paced at a constant frequency of 2 Hz by isolated rectangular pulses having 1 msec duration and 3 times threshold voltage, via bipolar platinum electrodes placed on the tissue. Each muscle was stretched to the length at which the force of contraction was maximal. The resting force (about 10 mN) was kept constant throughout the experiment. The twitch contractions were monitored on an EMG-1555 type oscilloscope and recorded on film and on a polygraph. The maximal contractile force was determined with and without drugs under steady state conditions. All drugs were added to the bath from concentrated stock solutions.

In some experiments, we studied the interaction between adenosine and antiarrhythmic agents in the presence of aminophylline, a specific

P_1-purinoceptor blocker, to get further information about the mechanism of these combined effects.

The transmembrane resting and action potentials were measured in a conventional way (Trautwein *et al.*, 1983) between an intracellular glass microelectrode filled with 3 M KCl solution and having a resistance of 5–10 MΩ and an extracellular Ag-AgCl electrode, both connected to a cathode follower and a DC amplifier (MIKI-1623). The recorded potentials were displayed on an EMG-1555 type oscilloscope and photographed on film (Kelemen *et al.*, 1968). The depolarization phase of the action potentials was differentiated with an analog differentiator and the maximum rate of rise ($\dot{V}_{max}$) was recorded as described previously by Papp and Vaughan Williams (1969). The preparations were electrically paced in the same way as described above. From the parameters of the transmembrane potentials, the resting membrane potential, the overshoot, the $\dot{V}_{max}$ and the action potential duration at 20 % and 90 % repolarizations were evaluated.

The drugs used in this study were adenosine (Boehringer, Mannheim, F.R.G.), quinidine sulphate (Alkaloida, Tiszavasvári, Hungary), verapamil (Knoll AG., Ludwigshafen, F.R.G.), aminophylline (Richter, Budapest, Hungary).

The experimental data presented are means ± S.E.M. The statistical significance of difference from the control was estimated using Student's *t*-test. A p value of less than 0.05 was considered significant.

Results

Effect of quinidine on the action potential and contractile force in atrial myocardium treated with adenosine

The electrophysiological and mechanical effects of adenosine and quinidine were studied in 5 isolated, electrically driven guinea-pig left atrial muscle preparations. Adenosine was used at a concentration of 10^{-5} M, which was found to be appropriate in former experiments (Mészáros *et al.*, 1984).

Table I summarizes the data and Fig. 1 shows original transmembrane action potential records obtained by intracellular microelectrode from atria (upper panels) and twitch contractions recorded at a slow sweep speed (lower panel) before and after 5 min exposure to adenosine followed by increasing doses ($10^{-5} - 5 \times 10^{-5}$ M) of quinidine. The control data of all experiments were pooled. Adenosine (10^{-5} M) was added to the bath solution after a preincubation period of 30 min. The effect of the drug was usually evident within 1 min after application and reached a steady state within 3 min. Adenosine markedly shortened both APD_{20} and APD_{90}, slightly hyperpolarized the cell membrane, slightly increased the $\dot{V}_{max}$ and

TA

Effect of quinidine (Quin) on adeno
and mechanical changes in a

Treatment	RP (mV)	OS (mV)	
Control	-86.4 ± 1.7	$+23.6 \pm 0.6$	19
AD 10^{-5} M	-91.6 ± 1.8*	$+23.0 \pm 0.8$	20
+Quin. 10^{-5} M	-87.0 ± 0.9*	$+23.0 \pm 1.0$	12
+Quin. 5×10^{-5} M	-82.6 ± 1.1*	$+21.4 \pm 1.6$	5

Mean ± S.E.M. of 5 experiments. Statistically significant differences fro
tial, OS: overshoot, $\dot{V}_{max}$: maximum rate of rise of action potential, APD
CF: contractile force (control value: 7.94 mN).

TABLE I

adenosine (AD)-induced electrophysiological es in atrial myocardium of guinea-pig

$\dot{V}_{max}$ (V/sec)	APD_{20} (msec)	APD_{90} (msec)	CF (%)
196.2 ± 1.8	18.0 ± 0.7	61.2 ± 0.3	100.0 ± 0.0
209.5 ± 1.3*	4.6 ± 0.6*	33.4 ± 0.3*	35.2 ± 0.3*
122.0 ± 1.7*	11.8 ± 0.8*	51.6 ± 0.9*	58.8 ± 1.6*
58.2 ± 1.3*	19.4 ± 0.7*	67.4 ± 0.2*	98.0 ± 2.0*

ces from the control and AD-treated represented by * $P < 0.05$. RP: resting poten- $APD_{20, 90}$: action potential duration at 20 % and 90 % repolarization, respectively,

reduced contractile force. Application of quinidine at a concentration of 10^{-5} M, which alone consistently decreases the $\dot{V}_{max}$ of the action potential without altering the resting membrane potential, prolongs the duration of the action potential, exerts a positive inotropic effect (Table II), and was able to antagonize the adenosine-induced changes in the electrical and mechanical activity of guinea-pig atrial myocardium. Incubation time was

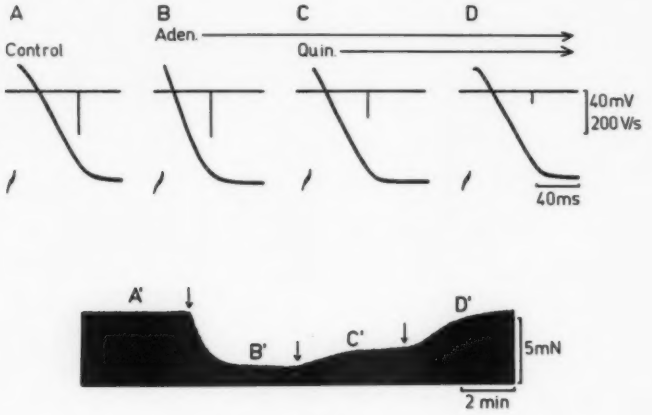

FIG. 1

Effect of quinidine (Quin) on the adenosine (Aden)-induced electrical and mechanical changes in left atrial myocardium of guinea-pig. Upper panels: Intracellularly recorded action potentials. $\dot{V}_{max}$ recordings (downward deflections of the zero potential line) are shifted to the right for better evaluation. Tracings were retouched where necessary to enhance reproduction. Lower panel: Contractions of a left atrial preparation recorded at a slow sweep speed. In this panel the letters indicate the times of recording of the action potentials shown in the upper panels. Note that quinidine antagonizes the action of adenosine.

20 min with each dose of quinidine, during which time period the drug-induced changes reached a new steady state. Quinidine decreased the $\dot{V}_{max}$, slightly depolarized the cell membrane but markedly prolonged the action potential duration, as well as increased the contractile force in atria treated with adenosine. This modulating effect became more pronounced when quinidine was added at a higher concentration (5×10^{-5} M) (see Table I and Fig. 1) which is capable of completely reversing the effect of adenosine (Fig. 3). As evidences have been reported in the literature for the hypothesis that adenosine exerts its effect via an interaction with an extracellular P_1-purinoceptor (Burnstock, 1976; Schrader *et al.*, 1977; Szentmiklósi *et al.*, 1979; 1980; Moody and Burnstock, 1982; Mészáros *et al.*, 1984), in one series of experiments we studied the interaction between

T

Effects of quinidine (Quin) and verapamil (
and relative tension in left

Treatment	RP (mV)	OS (mV)	
Control	-86.4 ± 1.7	$+23.6 \pm 0.6$	1
Quin. 10^{-5} M	-82.7 ± 1.1*	$+22.8 \pm 1.3$	1
Control	-86.4 ± 1.7	$+23.6 \pm 0.6$	1
Verap. 10^{-5} M	-85.4 ± 1.5	$+21.8 \pm 0.9$*	2

Mean $\pm$ S.E.M. of 5 experiments. Statistically significant differences fro
$\dot{V}_{max}$: maximum rate of rise of action potential, $APD_{20,90}$: action poten
force (control value: 7.94 ml).

TABLE II

...amil (Verap) on some electrophysiological parameters ...n left atrial myocardium of guinea-pig

	$\dot{V}_{max}$ (V/sec)	APD_{20} (msec)	APD_{90} (msec)	CF (%)
	196.2 ± 1.8	18.0 ± 0.7	61.2 ± 0.3	100.0 ± 0.0
	108.0 ± 2.4*	43.6 ± 0.4*	92.0 ± 1.2*	165.7 ± 2.8*
	196.2 ± 1.8	18.6 ± 0.7	61.2 ± 0.3	100.0 ± 0.0
	203.0 ± 2.9*	7.2 ± 0.8*	78.8 ± 1.8*	32.6 ± 3.1*

...es from the control represented by * $P < 0.05$. RP: resting potential, OS: overshoot, ... potential duration at 20 % and 90 % repolarization, respectively, CF: contractile

adenosine and quinidine on the contractile force in the presence of a specific P_1-purinoceptor blocker, aminophylline, in order to find out whether this receptor plays a role in the interaction. In these experiments, 5 isolated atria were pretreated with 5×10^{-5} M of aminophylline for 10 min, and then adenosine and quinidine were added in the bath solution at concentrations of 10^{-5} M and 5×10^{-5} M, respectively. The results are shown in Fig. 3. Adenosine had no negative inotropic effect when the specific P_1-purinoceptors were blocked. In case adenosine and quinidine were added together, only the action of quinidine became apparent.

Effect of verapamil on the action potential and contractile force in atrial myocardium treated with adenosine

The effect of verapamil (10^{-5} M) on the adenosine-induced changes in electrophysiological and mechanical activity was studied in 5 isolated, electrically driven left atrial myocardia of guinea-pigs. The experimental protocol was the same as described in the case of quinidine. The experimental data are summarized in Table III, and original transmembrane action potential recordings and twitch contractions are shown in Fig. 2. Verapamil, at a concentration of 10^{-5} M, which alone markedly

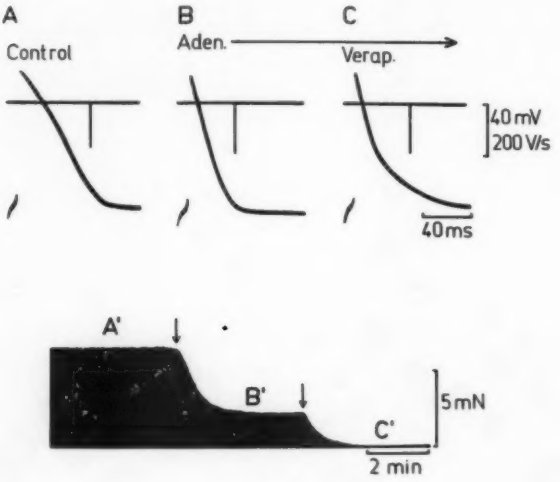

FIG. 2

Effect of verapamil (Verap) on the adenosine (Aden)-induced electrical (upper panels) and mechanical (lower panel) changes in left atrial preparations of guinea-pig. Note that verapamil potentiates the action of adenosine.

shortened the early repolarization phase (APD_{20}), but prolonged the late one (APD_{90}), and exerted a negative inotropic effect by blocking the slow inward Ca-current (Kohlhardt *et al.*, 1972), increased the cardiodepressant effect of adenosine or even stopped contractility—only action potentials could be recorded—i.e. it caused an excitation-contraction uncoupling in atria treated with adenosine.

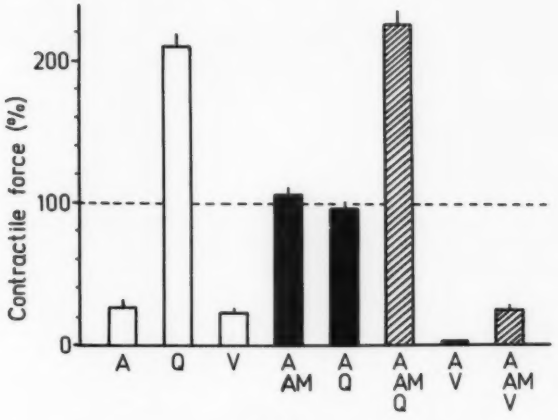

FIG. 3

Summary of the effect of adenosine (A; 10^{-5} M), quinidine (Q; 5×10^{-5} M), verapamil (V; 10^{-5} M), respectively, and in combinations with one another, as well as with a specific P_1-purinoceptor blocker, aminophylline (AM; 5×10^{-5} M) on the contractile activity of the left atrial preparations of guinea-pigs. Control value (7.94 mN, 100 %) is indicated by the broken line. Values represent means ± S.E.M. of 5 experiments. Open columns: action of one drug alone; black columns: action of two drugs together; shaded columns: action of three drugs together.

We also studied the interaction between adenosine and verapamil on the contractile activity in atria in which the specific adenosine receptors (P_1) were first blocked by aminophylline (5×10^{-5} M). As shown in Fig. 3, aminophylline-pretreatment (for 10 min) can attenuate the verapamil enlargement of the cardio-depressant effect of adenosine, i.e. under this condition, only the effect of verapamil becomes apparent.

Discussion

Agents which can modulate the adenosine-induced changes in the electrophysiological and mechanical properties of the myocardium, either at the P_1-purinoceptors level or via any other mechanisms (e.g. at the level

TA

Effect of verapamil (Verap) on the ad
and mechanical changes in a

Treatment	RP (mV)	OS (mV)	
Control	-86.4 ± 1.7	$+23.6 \pm 0.6$	19
AD 10^{-5} M	-91.6 ± 1.8*	$+23.0 \pm 0.8$	20
+ Verap. 10^{-5} M	-90.4 ± 2.1	$+21.6 \pm 0.7$	19

Mean $\pm$ S.E.M. of 5 experiments. Statistically significant differences fro
tial, OS: overshoot, $\dot{V}_{max}$: maximum rate of rise of action potential, $APD_{}$
CF: contractile force (control value: 7.94 mN).

TABLE III

the adenosine (AD)-induced electrophysiological
es in atrial myocardium of guinea-pig

$\dot{V}_{max}$ (V/sec)	APD_{20} (msec)	APD_{90} (msec)	CF (%)
196.2 ± 1.8	18.0 ± 0.7	61.2 ± 0.3	100.0 ± 0.0
209.5 ± 1.3*	4.6 ± 0.6*	33.4 ± 0.3*	35.2 ± 0.3*
198.8 ± 2.1*	2.6 ± 0.2*	40.8 ± 0.9*	0.0 ± 0.0*

ces from the control and AD-treated represented by * $P < 0.05$. RP: resting poten-
$APD_{20; 90}$: action potential duration at 20 % and 90 % repolarization, respectively,

of membrane ionic channels), are of interest not only as potential therapeutic agents but also as research tools. An understanding of the mechanism of action of these agents will undoubtedly lead to insights into the ionic mechanisms mediating the adenosine action. In the present study we wanted to investigate whether there is any interaction between adenosine, a well-known physiological metabolite released from cardiac cells mainly during myocardial hypoxia, and two widely used antiarrhythmic agents, quinidine (class 1 agent) and verapamil (class 4 agent; for classification, see Vaughan Williams, 1981) on the electrophysiological and mechanical properties of guinea-pig left atria. No attempt was made to relate effects of adenosine to those of beta-adrenoceptor blockers (class 2 agents), since these drugs, at concentrations needed to block the receptors, have no or class 1 agent-like electrophysiological actions (Hauswirth and Singh, 1979). The interaction between adenosine and BMA (class 3 agent) has been demonstrated in a previous study (Mészáros *et al.*, 1984). In accordance with our previous results (Mészáros *et al.*, 1984) and those of other authors (Hollander and Webb, 1957; De Gubareff and Sleator, 1965; Schrader *et al.*, 1975; Belardinelli *et al.*, 1979; Belardinelli and Isenberg, 1983), adenosine markedly shortened the action potential duration at all points of the repolarization phase, hyperpolarized the membrane, slightly increased $\dot{V}_{max}$ and exerted a negative inotropic effect. Although there are evidences suggesting that adenosine is able to block directly the slow inward Ca-channels (Schrader *et al.*, 1975; Belardinelli *et al.*, 1979), most authors agree that the drug rather increases the outward movement of K-ions during excitation, at least at relatively low concentrations (10^{-5} M, and below) (Goto *et al.*, 1977; Belardinelli and Isenberg, 1983; Hutter and Rankin, 1984; Mészáros *et al.*, 1984), which were also used in this study. This concept is also supported by the fact that selective Ca-channel blockers (e.g. verapamil, D-600, $MnCl_2$) in contrast to adenosine, shorten only the early phase of the repolarization, whereas they prolong the late repolarization phase (Kass and Tsien, 1975). This phenomenon is explained by a decrease in the slow inward Ca-current, which leads to a further decrease of the intracellular free Ca-concentration during the action potential and, consequently, decreases the outward K-current (Isenberg, 1975; Kass and Tsien, 1976). We assume that the adenosine-induced large and homogeneous shortening in action potential duration may be due to an increased K-conductance, i.e. the drug first increases the outward K-current, accelerates repolarization, and prevents the slow inward Ca-current from running its normal time course, which impairs the Ca-influx, thus producing a negative inotropic effect, as also described in the case of acetylcholine (Ten Eick *et al.*, 1976). The results obtained in experiments with aminophylline, a competitive antagonist of adenosine at the P_1-purinoceptor level in guinea-pig atrium (Burnstock, 1976; Szentmiklosi *et al.*, 1979; Moody and Burnstock, 1982) provide further

evidence for the hypothesis that adenosine requires freely available P_1-purinoceptors to exert its effect, and suggest that these receptors might play a role in the regulation of outward K-current, responsible for repolarization, similarly to the muscarinic receptors in the atrial muscle (Pappano and Inoue, 1984).

In the light of these considerations, it could be conceived that drugs modulating K-conductance, either directly or indirectly, may influence or even antagonize the development of the action of adenosine. We have demonstrated that a selective K-channel blocker, BMA (bromobenzoylmethyladamantylamine; class 3 agent) (Mészáros *et al.*, 1982) is capable of completely reversing the adenosine-induced shortening in action potential duration and reduction of contractile force in guinea-pig atria (Mészáros *et al.*, 1984). In the present experiments, quinidine, a membrane-stabilizing antiarrhythmic agent (class 1), which itself prolongs the action potential duration and exerts a positive inotropic effect in atria (see also Nawrath, 1981), was also able to reverse the adenosine-induced changes in electrophysiological and mechanical properties of guinea-pig left atria. Quinidine prolonged the action potential duration homogeneously, decreased the $\dot{V}_{max}$ and increased the amplitude of twitch contractions even in the presence of adenosine. A competition at the P_1-purinoceptors can be excluded, since it has been demonstrated by the determination of pA_2-values that there is a marked noncompetitive antagonism between adenosine and quinidine on the contractile activity of guinea-pig left atria (Szentmiklosi *et al.*, 1978). The most plausible explanation for this antagonism might be as follows: quinidine slows down the inactivation of the fast inward Na-current (Ducouret, 1976), which delays the onset of the repolarization and, consequently, widens the plateau phase (Brady and Woodbury, 1960). In this way, quinidine can improve the net Ca-influx and result in enlarged contractions in atria in which the plateau phase is relatively short. These electrophysiological events might account for the noncompetitive antagonism between adenosine and quinidine. It was also observed that aminophylline, a competitive antagonist of adenosine, potentiated the anti-adenosine effect of quinidine. We suppose that aminophylline, by blocking P_1-purinoceptors, decreases the number of freely available binding sites for adenosine and that, consequently, only the effect of quinidine can be demonstrated.

In another series of experiments, the effect of verapamil, a selective Ca-channel blocker, was studied in atrial myocardium pretreated with adenosine. It was found that verapamil, which is widely used as a therapeutic agent in the treatment of angina, arrhythmias and hypertension (Fleckenstein, 1983), increased the adenosine-induced shortening in the plateau phase of the action potential, and further diminished or even abolished the contractile activity. In other words, verapamil caused a complete excitation-contraction uncoupling in atria pretreated with adenosine.

The results call attention to the fact that application of verapamil may be dangerous in the ischemic heart muscle, at least in atria, taking into account that adenosine is released in large amounts during hypoxia. Curtis *et al.* (1984) have demonstrated that verapamil produces a dose-dependent increase in non-arrhythmic deaths in rats in which ischemic heart failure was induced by occlusion of a coronary artery.

In summary, our results indicate that the adenosine-induced shortening in the action potential duration may be due to a large increase in the outward K-current, and that the P_1-purinoceptors are also involved in this mechanism, which impairs the contractile force of atrial myocardium. Quinidine, which prolongs the plateau phase of the action potential, is capable of completely antagonizing the effects of adenosine, whereas verapamil, which blocks the slow Ca-channels directly, potentiates the adenosine action.

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Effect of Intrathecal and Intracarotid Administration of Ketamine on Blood Pressure and Heart Rate in Rats

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Abstract—Intrathecal and intracarotid effects of ketamine were investigated in anaesthetized rats. Ketamine was administered in the subarachnoid space in doses ranging from 300 to 1500 $\mu g.kg^{-1}$. In these doses ketamine produced a short-lasting hypotension and bradycardia which may be due to inhibition of sympathetic outflow from thoraco-lumbar region. On the other hand when ketamine ($5\ mg.kg^{-1}$) was injected into the cerebral circulation it produced a rise in blood pressure and heart rate, probably through a cholinergic mechanism.

Introduction

Ketamine is an intravenous anaesthetic agent which provides good analgesia for surgical patients (McCarthy *et al.*, 1965). Following intravenous injection, tachycardia and increases in systemic arterial blood pressure are often observed. These changes are accompanied by decreases in overall systemic vascular resistance (Roberts, 1980).

In experimental animals, ketamine has been shown to produce an increase in blood pressure in rats (Chen, 1973) and in dogs (McCarthy *et al.*, 1965, Dowdy and Kaya, 1968). On the other hand, falls in blood pressure following ketamine have been observed in rats (Chang *et al.*, 1969) and rabbits (McGrath *et al.*, 1975, Dhasmana *et al.*, 1984). Some workers (Chen, 1973, Dowdy and Kaya, 1968) have noted a biphasic response. Moreover, when injected into the cerebral circulation, ketamine produces an immediate increase in blood pressure, heart rate and cardiac output

(Ivankovich *et al.*, 1974) thus suggesting a direct stimulation of CNS structures.

The purpose of the present investigation was to analyze the effects of ketamine on blood pressure and heart rate when delivered directly into the subarachnoid space and into the cerebral circulation in the anaesthetized rat.

Methods

Wistar rats of either sex, weighing between 250 and 350 g, were used in the present investigation. Anaesthesia was induced with halothane (1–2 %) vaporised in nitrous oxide and oxygen (2:1). Following tracheotomy, the animals were ventilated with halothane (0.3–0.5 %), nitrous oxide and oxygen using a Rosche Baby Kontrol ventilator; end expiratory carbon dioxide, monitored on a Gould Godart capnograph Mark III, was maintained at 4.5–5 volumes per cent.

Neuromuscular blockade was maintained using a continuous infusion of pancuronium bromide (90 $\mu g.kg^{-1}.hr^{-1}$) via a Braun Melsungen infusion pump. The femoral artery was cannulated and attached to a Gould Statham P23 ID pressure transducer. Arterial blood pressure was displayed via a Grass 7D recorder and ink writing polygraph. The femoral vein was cannulated and used for the administration of drugs. The heart rate was recorded using a tachograph (Model 7P4D Grass instrument) which was triggered by lead II of the electrocardiogram and displayed on the oscillograph.

Intrathecal catheter

In order to observe the peripheral cardiovascular effects of ketamine, the drug was administered intrathecally in the thoracic or lumbar region (Yaks and Rudy, 1976). To achieve this, a thin polyethylene tube (external diameter 0.61 mm) had been introduced one hour earlier (under anaesthesia) into the subarachnoid space of the spinal cord. Introduction had been performed via the cisterna magna and the cannula was advanced into the lumbar region—a distance of 7.5 cm. The total length of the tube was 14 cm with a volume capacity of 10 μl. The rats had been allowed to recover and only those showing no subsequent neurological damage were included in the study.

The doses of ketamine chosen for intrathecal administration have no significant effect on the blood pressure and heart rate when administered intravenously. The drugs were injected in volumes of 5 μl followed by saline 10 μl. The effects of a single intrathecal dose of ketamine were observed for 30 min. Following recovery, the next dose was administered. In this

manner, four doses of ketamine (300, 600, 900 and 1500 $\mu g.kg^{-1}$) were administered in one experiment. A group of 7 rats was used as control and they received similar volumes of saline via their intrathecal catheters.

Intracarotid catheter

For injecting ketamine into the cerebral circulation, a polyethylene catheter was introduced into one of the carotid arteries. Blood pressure was recorded from one femoral artery, and heart rate through electrocardiogram lead II as described above. Anaesthesia was maintained as mentioned earlier.

For antagonising the pressor response obtained after intracarotid injection of ketamine, a number of antagonists (atropine sulphate 5 $mg.kg^{-1}$ i.c., propranolol 1 $mg.kg^{-1}$ i.c., dibenzyine 1 $mg.kg^{-1}$ i.c., ketanserin 2 $mg.kg^{-1}$ i.c., diazepam 2 $mg.kg^{-1}$ i.c. and naloxone 2 $mg.kg^{-1}$ i.c.) were administered.

Results

Intrathecal ketamine

The effects of intrathecal ketamine were observed at 1, 2, 3, 5, 10, 20 and 30 min intervals. Immediately after administration of ketamine intrathecally there was an acute fall in blood pressure, as can be observed in Table I. Ketamine, in doses ranging from 300 to 900 $\mu g.kg^{-1}$ produced an immediate and highly significant decrease in blood pressure. This gradually rose again and, after 10 min, the level was no longer significantly different from the control values. However, the highest dose of ketamine (1500 $\mu g.kg^{-1}$) caused a significant fall in blood pressure for 20 min. Intrathecal saline had no significant effect on systemic blood pressure.

In Table II the effects of intrathecal ketamine administration on heart rate are presented. As can be seen, a dose of 300 $\mu g.kg^{-1}$ produced a significant decrease in heart rate lasting for 30 min. However, with the subsequent higher doses, the decrease in heart rate after ketamine was observed for only 5 min. The injection of saline into the subarachnoid space did not produce any significant effect on the heart rate.

Intracarotid ketamine

Ketamine was injected into the carotid artery in a dose of 5 $mg.kg^{-1}$. It produced an immediate rise in blood pressure from 124 ± 4 to

TA

Effects of intrathecal administration of ketamine on mea

Dose of ketamine ($\mu g.kg^{-1}$)	control	1 min	2 min
Saline	129 ±3 (7)	131 ±3 (7)	131 ±4 (7)
300	132 ±2 (11)	94*** ±7 (11)	104*** ±5 (11)
600	128 ±2 (11)	75*** ±5 (11)	88*** ±6 (11)
900	125 ±3 (11)	75*** ±6 (11)	80*** ±6 (11)
1500	129 ±3 (11)	75*** ±6 (11)	78*** ±5 (11)

Significant difference from control is indicated by asterisks. * = $p < .05$,
The number of observations in each group is given in brackets.

TABLE I

...n mean arterial pressure (mm Hg ± SEM) in anaesthetised rats

	3 min	5 min	10 min	20 min	30 min
	134	134	129	131	129
	±5	±4	±3	±3	±3
	(7)	(7)	(7)	(7)	(7)
*	114***	124*	128	127	126
	±5	±4	±3	±4	±3
	(11)	(11)	(11)	(11)	(11)
*	99**	112**	125	125	126
	±5	±5	±3	±3	±3
	(11)	(11)	(11)	(11)	(11)
*	95**	113*	126	126	126
	±7	±5	±3	±3	±2
	(11)	(11)	(11)	(11)	(11)
*	86***	98***	113**	123*	133
	±6	±5	±5	±2	±4
	(11)	(11)	(11)	(11)	(11)

$^{*}p<.05$, $^{**}p<.01$, $^{***}p<.001$.

Effects of intrathecal administration of ketamine

Dose of ketamine ($\mu g.kg^{-1}$)	control	1 min	2 min
Saline	426 ±13 (7)	426 ±13 (7)	429 ±14 (7)
300	435 ±8 (11)	412** ±9 (11)	406*** ±10 (11)
600	423 ±9 (11)	401*** ±11 (11)	382*** ±10 (11)
900	420 ±8 (11)	386*** ±9 (11)	383*** ±8 (11)
1500	420 ±7 (10)	395* ±9 (10)	383** ±8 (10)

Significant difference from control is indicated by asteriks. * = p<.
The number of observations in each group is given in brackets.

TABLE II

mine on heart rate (beats per min ± SEM) in anaesthetised rats

in	3 min	5 min	10 min	20 min	30 min
9	429	431	429	431*	430
4	±14	±15	±14	±15	±14
)	(7)	(7)	(7)	(7)	(7)
5***	410***	415**	420*	422*	423*
0	±9	±9	±10	±9	±9
)	(11)	(11)	(11)	(11)	(11)
2***	389***	402**	415	416	418
0	±10	±11	±10	±10	±9
)	(11)	(11)	(11)	(11)	(11)
3***	390***	399*	416	418	422
8	±8	±9	±9	±10	±7
)	(11)	(11)	(11)	(11)	(11)
3**	378**	388*	396	410	416
8	±10	±11	±14	±12	±10
0)	(10)	(10)	(10)	(10)	(10)

= $p < .05$, ** = $p < .01$, *** = $p < .001$.
ts.

TA

Effect of intracarotid injection of ketan
on blood pressure

Pretreatment	Mean blood pressure (mm Hg) After drug treatment	Aft
Saline (n = 6)	124 ± 4	
Diazepam (n = 6) (2 mg.kg^{-1})	154 ± 6	
Ketanserin (n = 6) (1 mg.kg^{-1})	94 ± 8	
Naloxone (n = 6) (2 mg.kg^{-1})	126 ± 10	
Propranolol (n = 6) (1 mg.kg^{-1})	100 ± 7	
Atropine (n = 6) (5 mg.kg^{-1})	133 ± 6	
Dibenzyline (n = 6) (1 mg.kg^{-1})	83 ± 9	

* Significant ($p < 0.05$) difference from control. Results are expressed

TABLE III

f ketamine before and after pretreatment of drugs
essure and heart rate in rats

Mean heart rate (beats.min^{-1}) After drug treatment	Ketamine (5 mg.kg^{-1} i.c.) Mean blood pressure (mm Hg)	Mean heart rate (beats.min^{-1})
372 ± 12	194 ± 4*	383 ± 12*
402 ± 19	190 ± 6*	410 ± 17*
342 ± 18	126 ± 11*	367 ± 17*
393 ± 16	167 ± 18*	404 ± 16
356 ± 10	150 ± 5*	350 ± 10
438 ± 12	146 ± 11	445 ± 12
358 ± 15	94 ± 11	346 ± 11

essed as mean ± standard error of mean.

149 ± 4 mm Hg ($\pm$SEM) ($p < 0.05$) and an increase in heart rate from 372 ± 12 to 383 ± 12 ($\pm$SEM) ($p < 0.05$) (Table III).

After diazepam ($2\ mg.kg^{-1}$) administration there was no significant change in control blood pressure and heart rate. Similar effects were observed with naloxone, atropine and propranolol. There was a significant fall in blood pressure and heart rate after ketanserin ($1\ mg.kg^{-1}$) and dibenzyline ($1\ mg.kg^{-1}$) administration. The ketamine-induced pressor response was significantly antagonized following administration of atropine and dibenzyline (Table III).

Discussion

Ketamine is an intravenous anaesthetic agent which easily passes the blood brain barrier and, on reaching the central nervous system, produces surgical anaesthesia. In humans, increases in heart rate are accompanied by an increase in blood pressure and cardiac output (Roberts, 1980) whereas in rats, high doses of intravenous ketamine cause a fall in blood pressure (Clanachan *et al.*, 1976).

In the present investigation, intrathecal ketamine produced a depressor effect on blood pressure and heart rate. This indicates the presence of a spinal site of action of ketamine. This could be due to inhibition of sympathetic outflow from the spinal cord, especially in view of the bradycardia that is produced. This would indicate concomitant block of the cardioaccelerator component of the thoraco-lumbar outflow.

In this context, it is of considerable interest to remember that similar doses of intrathecal ketamine have been found to produce an analgesic effect; this effect could be antagonized by naloxone (Ahuja, 1983). We however (unpublished observations), were unable to antagonize the cardiovascular effects of intrathecal ketamine with naloxone. Narcotic analgesics are known to produce hypotension and bradycardia following intracisternal administration (Laubie *et al.*, 1979) and it may also be possible that ketamine reaches the brain stem following intrathecal administration. This is further substantiated by the fact that intracisternal administration of ketamine results in hypotension and bradycardia (unpublished observations).

Following intracarotid injection of ketamine, there occurred a significant rise in blood pressure and tachycardia which is in agreement with previous published results (Ivankovich *et al.*, 1974). Pretreatment with diazepam, or given together with ketamine, produces no cardiovascular disturbances (Thorsen and Gran, 1980). However, in our experiments pretreatment with diazepam failed to antagonize the pressure and tachycardiac response obtained with ketamine. Furthermore, a serotonin blocker (ketanserin) and a beta blocker (propranolol) failed to antagonize the pressor response of

ketamine, but the tachycardiac response of ketamine was prevented by pretreatment with naloxone and propranolol. However, we have observed that the blood pressure and tachycardic responses of ketamine are antagonized by atropine, thus suggesting an involvement of the cholinergic system in the CNS. Moreover, blockade of pressor response of ketamine by dibenzyline indicates an involvement of the peripheral sympathetic system.

This is further substantiated by the fact that ketamine increases catecholamine concentration in the peripheral blood (Kumar *et al.*, 1978) and at the same time blocks the uptake of noradrenaline (Salt *et al.*, 1979). Thus it appears that the initial pressor response of ketamine is through a stimulation of the central nervous system and the decrease in blood pressure via a spinal site of action.

In conclusion, this paper has demonstrated the existance of spinal and cerebral sites of action of ketamine. The spinal site is responsible for producing hypotension and bradycardia, whereas the cerebral site is responsible for rise in blood pressure and tachycardia.

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Clonidine and Enphenamic Acid Interaction Study in Dogs

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Abstract—The drug interaction between clonidine (Cl) and enphenamic acid (En.A.), a newer non-steroidal anti-inflammatory drug (NSAID) was studied in anaesthetized dogs. Prior administration of En.A. in animals modified the blood pressure response to clonidine. Thus En.A., administered 1 hr before clonidine, potentiated the hypertensive response and blocked the subsequent hypotensive response to clonidine. Intracerebroventricular administration of En.A. did not affect the usual blood pressure response to clonidine. Similarly, this interaction was not observed in reserpinized animals and it was only partially blocked in normal dogs, treated with tolazoline. It is concluded that En.A. interferes with blood pressure responses to clonidine and that this interaction is probably of a peripheral, vascular, nature.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed in practice. These drugs are likely to be administered to patients who might be taking other drugs such as antihypertensive agents and therefore may give rise to undesirable effects due to drug interaction. Thus, indomethacin is known to cause a decrease in sodium and water excretion in patients with high blood pressure and may antagonize the antihypertensive effect of furosemide (Patak *et al.*, 1975), thiazide (Watkin *et al.*, 1980), propranolol and oxprenolol (Durao *et al.*, 1977; Salvetti *et al.*, 1982). Ibuprofen, an NSAID, has been reported to reverse the antihypertensive effect of pindolol (Reid, 1981). Indomethacin has also been reported to reverse the antihypertensive effect of pindolol in rabbits (Durao and Rico, 1977) and of hydralazine and minoxidil in dogs (Hausler and Gerold, 1979).

Enphenamic acid (En.A.) is a new anthranilic acid derivative marketed for the treatment of pain and inflammation. Recently, we reported a drug interaction following administration of En.A. to a hypertensive patient taking clonidine (Kshirsagar *et al.*, 1983). This experimental study, therefore, was undertaken to examine the interaction between clonidine and enphenamic acid and the possible site and mechanism of this interaction. A preliminary communication on this problem has been reported previously (Dalvi *et al.*, 1981).

Methods

Experiments were carried out in mongrel dogs and domestic cats of either sex. Animals were anaesthetized with 35 mg/kg of pentobarbital sodium administered intravenously (i.v.) in dogs and intraperitoneally (i.p.) in cats. Cannulation of the femoral vein was done for administration of drugs. The carotid artery was cannulated and the mean blood pressure (B.P.) recorded on a smoked drum, using a mercury manometer and a pointer. Various sequences of drugs and time intervals of administration were tried to study the interaction. Four dogs were used for each sequence and the results were compared with a control group.

Experiments were also carried out in dogs of either sex to elucidate the probable site and mechanism of interaction. Five dogs were reserpinized by injecting intramuscularly 0.35 mg/kg of reserpine twice daily for two successive days; the animals were used on the third day (Ghosh, 1971). Pentobarbital sodium was given in a dose of 20 mg/kg i.v. in reserpinized dogs. Depletion of catecholamines was confirmed by administration of tyramine 300 μg/kg i.v., a dose found adequate to produce pressor responses in normal animals; reserpinized animals in which the tyramine response was absent, were selected for further study. Intracerebroventricular cannulation (i.c.v.) was done by the method described by Feldberg and Sherwood (1953).

Clonidine hydrochloride (Cl) was used in a dose of 5 μg/kg i.v. and enphenamic acid (En.A.) in a dose of 10 mg/kg i.v., as a fine suspension in carboxymethyl cellulose (C.M.C.). These doses were calculated from recommended daily human doses according to a conversion factor as described by Paget and Barnes (1964). The control group received C.M.C. (vehicle).

A paired *t*-test was applied for statistical analysis of the data. The results are expressed as means $\pm$ S.E.

Results

Effect of Clonidine on B.P. in anaesthetized dogs and cats

In dogs, i.v. administration of clonidine in a dose of 5 μg/kg caused an initial transient B.P. rise of 16.8 ± 1.854 mm Hg, followed by a gradual fall of 27.2 ± 3.72 mm Hg below the basal level and bradycardia. The heart rate decreased from 129.6 ± 3.429 to 104.8 ± 4.27 per min. Maximum blood pressure fall was observed at 47 ± 1.22 min and hypotension persisted for 122.5 ± 2.26 min. Blood pressure then showed a gradual recovery towards basal levels.

In cats, i.v. administration of 5 μg/kg of clonidine caused a similar initial rise in B.P. as in dogs but the fall was minimal and not consistent. Similar effects have been reported by Laverty (1973). Therefore, all subsequent experiments were carried out in dogs.

Effect of enphenamic acid and clonidine on blood pressure in anaesthetized dogs

To study the drug interaction, the two drugs were administered in various sequences and at different time intervals in three groups. Four dogs were used in each group and the results in each group were compared with those in the control group. Administration of vehicle alone in the control group had no effect on blood pressure. Further, its administration before or after clonidine did not modify the blood pressure responses to clonidine.

In the first group, Cl was administered 5 min and 1 hr after En.A. The animals of the control group received vehicle instead of En.A. Clonidine, given 1 hr after En.A., produced a significantly larger ($p < .05$) rise in initial B.P. (34.5 ± 3.89 mm Hg) than that observed following Cl injection 5 min after En.A. (16.6 ± 1.5 mm Hg). The hypotensive response to Cl in dogs treated with En.A. or vehicle, however, remained similar (Fig. 1).

The second group of animals was given Cl and the usual hypertensive (20.5 ± 2.06 mm Hg) and hypotensive (22.0 ± 3.74 mm Hg) responses to clonidine were recorded. One hr after Cl, these animals received En.A., which resulted in a gradual reversal of the hypotensive response, which was, however, not statistically significant. Repeating the dose of Cl half an hr after En.A. produced a significantly greater ($p < .01$) initial rise in mean B.P. (32 ± 2.708 mm Hg), followed by a reduction of the hypotensive response (18 ± 3.915 mm Hg) which was, however, not statistically significant. The hypotensive response to a repeat dose of Cl in these animals was lower than that observed in the control group (Fig. 2).

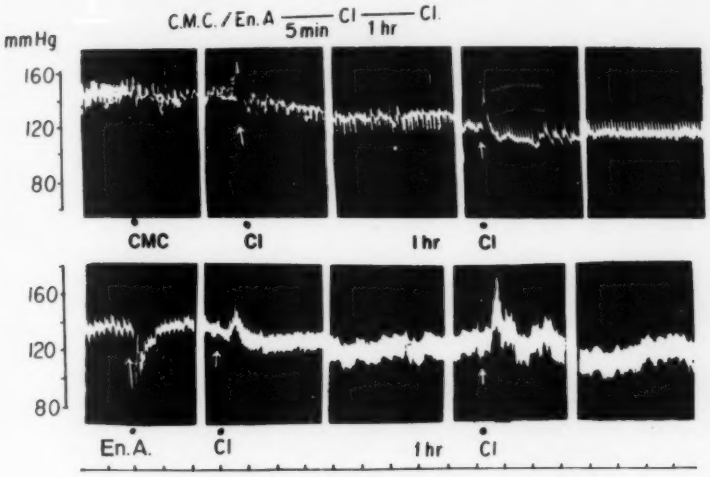

FIG. 1

Demonstration of interaction between clonidine (Cl 5 μg/kg) and enphenamic acid (En.A. 10 mg/kg) in anaesthetized dogs. Cl is injected 5 min and 1 hr after C.M.C. (vehicle) or En.A. (Time scale |__| = 1 min).

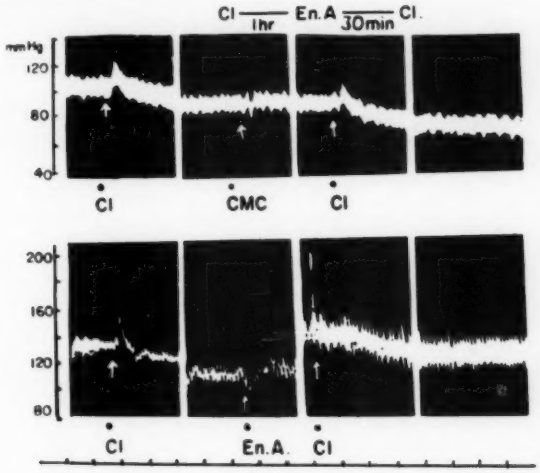

FIG. 2

Demonstration of interaction between Cl (5 μg/kg) and En.A. (10 mg/kg) in anaesthetized dogs. Cl is injected 1 hr before and 30 min after C.M.C. (vehicle) or En.A. (Time scale |__| = 1 min).

In the third group of dogs, injections of Cl, En.A. and Cl were given at 1 hr intervals. En.A. administration, 1 hr after clonidine, caused a reversal of the hypotensive response in all animals studied, whereas the B.P. increased from 117.2 ± 3.136 mm Hg to 128.8 ± 3.262 mm Hg ($p < .01$). Repeating the dose of clonidine at this stage caused a greater ($p < .01$) initial rise in B.P. (44.8 ± 2.72 mm Hg) as compared to the control response (20.8 ± 2.576 mm Hg) in the same animals. The subsequent hypotensive responses to clonidine were 7.6 ± 1.433 mm Hg, compared to pretreatment responses of 22.8 ± 1.959 mm Hg ($p < .05$). A statistically significant reduc-

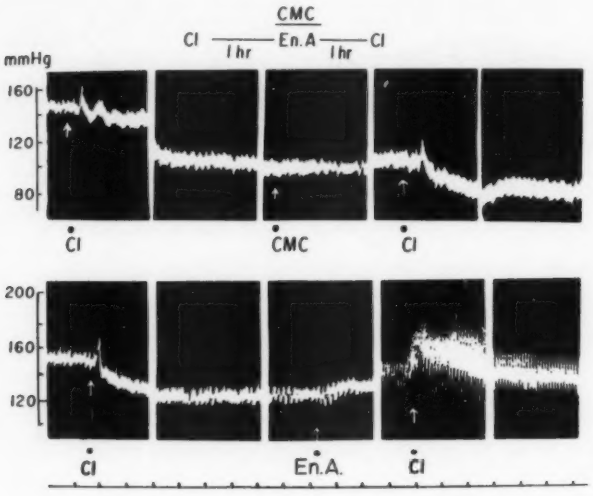

FIG. 3

Demonstration of interaction between Cl (5 μg/kg) and En.A. (10 mg/kg) in anaesthetized dogs. Cl is injected 1 hr before and 1 hr after C.M.C. (vehicle) or En.A. (Time scale |__| = 1 min).

tion in heart rate occurred following repeat dose of clonidine (95.2 ± 2.245 and 80.4 ± 3.31) as compared to the first dose (103 ± 2.059 and 106 ± 5.946) in the control and En.A. treated group, respectively. The bradycardia observed when repeating the dose of clonidine in the En.A. treated group was significantly greater ($p < .02$) than the bradycardia in the control group. Since the interaction between Cl and En.A. was marked in this group, this sequence of drug administration was used in further experiments to elucidate the possible mechanism of interaction (Fig. 3).

Effect of En.A. on B.P. responses to epinephrine (E) and norepinephrine (NE)

The anaesthetized dogs were given E (3 μg/kg) and NE (3 μg/kg) i.v. and blood pressure responses were recorded. The animals then received En.A. i.v. in a dose of 10 mg/kg. Repeating the administration of E and NE at 1 hr following En.A., showed a response which persisted for a significantly longer period of time ($p < .02$; E 1.825 ± 0.292 min, NE 2.225 ± 0.193 min as compared to control responses E 0.925 ± 0.110 min, NE 1.175 ± 0.103 min) (Fig. 4).

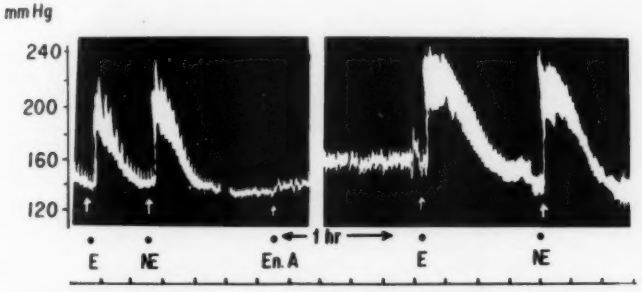

FIG. 4

Experiments to elucidate site and mechanism of Cl and En.A. interaction in anaesthetized dogs. Epinephrine (E) 3 μg/kg, Norepinephrine (NE) 3 μg/kg. (Time scale |__| = 1 min).

Effect of alpha-adrenoceptor blocker on Cl and En.A. interaction

After confirming the interaction between Cl and En.A., alpha-adrenoceptor blockade was induced in five anaesthetized dogs by slow i.v. administration of 4 mg/kg of tolazoline. Alpha-adrenoceptor blockade was confirmed by noting the absence of responses to injected epinephrine in these animals. The hypertensive response to a subsequent dose of clonidine was partially abolished.

Effect of reserpinization on the Cl and En.A. interaction

Reserpinized animals had a low basal blood pressure; responses to carotid occlusion and tyramine were absent. In five reserpinized dogs, Cl, En.A. and Cl were administered at 1 hr intervals. The hypertensive response to the first dose of clonidine was greater than that observed in normal animals. However, there was no statistically significant difference between blood pressure responses to clonidine given before and after En.A. (Fig. 5).

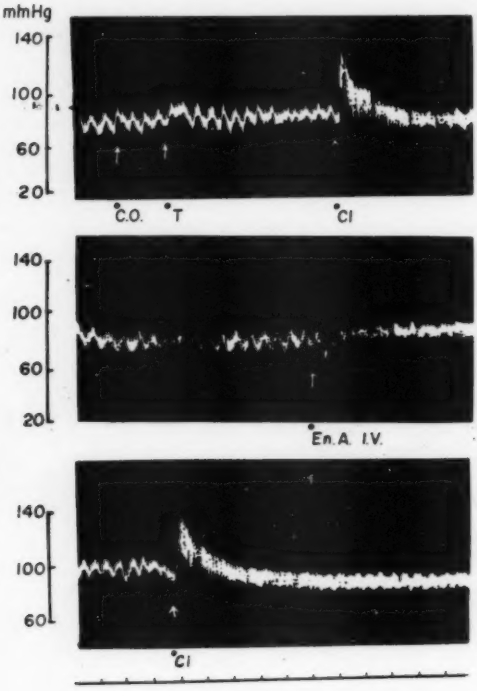

FIG. 5

Experiments to elucidate site and and mechanism of Cl and En.A. interaction in reserpinized, anaesthetized dogs. Reserpinization (reserpine 0.35 mg/kg B.D. i.m. for 2 days) is confirmed by the absence of response to carotid occlusion (C.O.) and tyramine (T. 300 μg/kg), Cl-En.A. interaction does not occur. (Time scale |___| = 1 min).

Effect of intracerebroventricular (i.c.v.) En.A. on the response to intravenous clonidine

Intracerebroventricular cannulation was carried out in 5 normal dogs and usual B.P. responses to i.v. clonidine were recoded. Animals then received En.A. (1 mg/kg i.c.v.). There was no change in B.P. following administration of En.A. Repeat dose of i.v. clonidine after half an hr revealed no statistically significant change in both the initial rise in B.P. and the subsequent hypotensive response to Cl, in all animals. However, administration of En.A. i.v., in the usual dosage, modified in these animals the blood pressure responses to clonidine, injected after 1 hr, as observed in earlier experiments (Fig. 6).

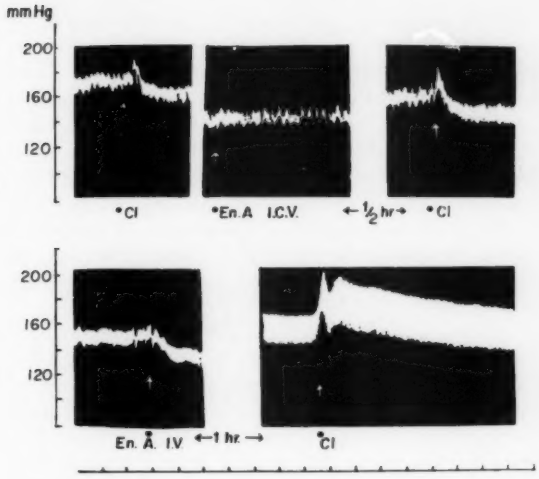

FIG. 6

Experiments to elucidate the site and mechanism of Cl and En.A. interaction in anaesthetized dogs. Cl-En.A. interaction does not occur when En.A. (1 mg/kg) is administered intracerebroventrically (i.c.v.). However, it is elicited in the same animal when En.A. (10 mg/kg) is injected i.v. (Time scale |___| = 1 min).

Discussion

The present study in dogs confirms that enphenamic acid administration after clonidine reverses its hypotensive effect. It also enhances the initial hypertensive response to clonidine and blocks the subsequent hypotensive response to repeat dose of clonidine.

The mechanism of the drug interaction between NSAID and antihypertensive drugs is not known. Indomethacin is a PG synthetase inhibitor. Prostaglandins, particularly E2, I2 and A2, have been shown to enhance sodium excretion and produce vasodilatation. It has been postulated that the effect of indomethacin on blood pressure and sodium excretion may be due to its PG synthetase inhibiting activity (Patak *et al.*, 1975; Durao *et al.*, 1977; Salvetti *et al.*, 1982). Indomethacin, administered to sodium depleted hypertensive patient taking propranolol, did not modify the hypotensive effect of propranolol (Frolich *et al.*, 1979).

Enphenamic acid also inhibits PG synthetase, but unlike indomethacin it does not cause a reduction of sodium excretion (Kshirsagar *et al.*, 1980, 1981). The hypertensive patient who developed clonidine and En.A. interaction, had a urinary output and sodium excretion similar to that

observed in the control period. Hence, a possible interaction through sodium retention involving renal prostaglandins is considered unlikely.

Clonidine is a central alpha-adrenoreceptor stimulant. Central alpha-adrenoreceptor stimulation produces a decrease in sympathetic tone and a fall in blood pressure (Schmitt *et al.*, 1967; Kobinger and Walland, 1967; Schmitt *et al.*, 1968). Clonidine, after i.v. administration, also causes an initial transient rise in blood pressure due to a stimulation of peripheral alpha-adrenoceptors (Hausler, 1973; Starke and Altmann, 1973; van Zwieten, 1973). It is unlikely that En.A. interferes with the central action of clonidine since administration of En.A. intracerebroventricularly did not modify the initial rise in blood pressure and the subsequent hypotension following intravenous clonidine.

It is well known that prostaglandins exert a modulating action on the sympathetic nervous system at both the pre- and post-synaptic level (Horton, 1973). Sympathetic nerve stimulation or infusion of noradrenaline leads to prostaglandin release (Hedqvist, 1970; Brody and Kadowitz, 1974). The released PGE_2 is the most important modulator of the pressor system which operates within the vascular wall. It attenuates constriction induced by administration of pressor hormones and sympathetic nerve stimulation not only by counteracting vasoconstriction, but also by inhibiting the release of norepinephrine from nerve endings (Hedqvist, 1970; McGiff and Spokas, 1981; McGiff, 1981). In our study, En.A. potentiated the action of E and NE. Furthermore, the interaction between Cl and En.A. was absent in reserpinized dogs. This suggests that En.A., a PG synthetase inhibitor, blocks the synthesis of modulating PG in the blood vessels, resulting in an uninhibited release of NE from sympathetic neuronal endings. This potentiates the E and NE responses as well as the hypertensive rise observed following clonidine and counteracts its subsequent hypotensive effect. In reserpinized animals, the neuronal catecholamine content was depleted and hence the interaction did not occur. A peripheral site of action is further supported by the finding that the overstimulation of peripheral alpha-adrenoreceptor was partially blocked by an alpha-adrenoceptor blocker, tolazoline. Similar drug interactions between clonidine and some other NSAIDs have been observed by us in experimental animals (unpublished data).

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Relationship between Plasma and Brain Concentrations of Denzimol and its Anticonvulsant and Neurotoxic Effects in the Rat

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Abstract—The relationship between plasma, brain and cerebellum concentrations of Denzimol® a new anticonvulsant agent, and its anticonvulsant and neurotoxic effects were studied in the rat. Sixty minutes after oral administration, a high brain/plasma concentration ratio of 10:1 was found. Mean plasma, brain and cerebellum half-lives of the drug were 8.4, 7.1 and 9.2 hr, respectively. The anticonvulsant activity of the drug was examined by maximal electroshok seizures (MES). Neurotoxicity was determined both by the rotarod performance test and by behavioural observation. Denzimol was effective against MES, in the ranges of 0.8–5 μg/ml and 10–50 μg/ml for plasma and brain concentrations, respectively, without causing clear signs of neurological toxicity. It is concluded that the anticonvulsant and neurotoxic effects of denzimol are correlated to its plasma and brain concentrations.

Introduction

The importance of monitoring plasma concentrations of antiepileptic drugs in order to obtain better therapeutic effects from treatment has been widely emphasized (Woodbury *et al.*, 1972; Kutt and Penry, 1974; Lund, 1974). The clinical ranges of plasma concentrations necessary for controlling epilepsy without causing toxic effects have been established for various standard drugs (Schneider *et al.*, 1975; Eadie, 1976; Gardner-Thorpe *et al.*, 1977; Pippenger *et al.*, 1978).

Recently, it has been demonstrated that, in animals, both the anticonvulsant and neurotoxic effects of the anticonvulsant drugs phenytoin, carbamazepine, phenobarbital, AD-810 and valproate are more closely correlated with their plasma concentrations than with the dosage

administered (Masuda *et al.*, 1979; Tulloch *et al.*, 1982). Furthermore, the same studies confirmed that the effective concentration ranges of these drugs in animal plasma were comparable to their reported therapeutic ranges in the plasma of epileptic patients. These studies are important not only for a better understanding of the pharmacological and toxicological properties of established antiepileptic drugs, but also for predicting clinical plasma concentrations of candidate anticonvulsant drugs, based on the results obtained in animal models.

Denzimol is a new potential antiepileptic drug (Nardi *et al.*, 1981). The anticonvulsant and pharmacotoxicological properties of denzimol have been previously described (Graziani *et al.*, 1983a, b, c). In the present study, we have examined the relationship between plasma, brain and cerebellum concentrations of denzimol and its anticonvulsant effects against maximal electroshock seizures. We have also studied at which tissue concentration the neurological toxicity of denzimol can be observed.

Methods

Animals

Female albino Sprague-Dawley rats (Grl:CD° (SD) BR) weighing 130–160 g were used in all experiments.

Animals were maintained on a 12 hr light-dark schedule at 22–24°C and were fasted overnight before the treatments.

Time-course of drug levels and anticonvulsant activity

Nine groups of 10 rats each were treated orally with 30 mg/kg of denzimol, suspended in 10 ml/kg 0.25 % of carboxymethylcellulose (CMC). Ten control rats were treated orally only with the vehicle. The drug-treated groups were tested for the anticonvulsant effect against maximal electroshock seizures (MES, according to the method of Swinyard, 1952) at 5, 15, 30, 60, 120, 240, 360, 480 and 720 min after administration. To each group, a predetermined experimental time was assigned. Seizures were elicited by applying a 130 mA 150 Hz 2 sec pulse via ear electrodes.

Complete abolition of the hindlimb tonic extensor components of seizures were taken as evidence of anticonvulsant activity. Blood was obtained by means of decapitation immediately after MES on 50 % of the animals of each group, and collected in heparinized tubes. After centrifugation, the plasma was stored at −20° C until analysis. Brain and cerebellum were immediately removed and stored at −20° C.

Dose-response relationship of anticonvulsant activity

Eight dose groups and one control group of 10 rats each were used. Animals were treated orally with 1, 2, 4, 8, 16, 32, 64 and 128 mg/kg of denzimol suspended in 10 ml/kg of 0.25 % CMC.

One hour after treatment, the anticonvulsant activity against MES (performed as previously described) was tested.

The presence of tonic extension of fore (TFP) and hind paws (THP) was recorded.

Visual observation of ataxia (ATA) was also performed and scored as follows: 0 (no ATA), 1 (doubtful ATA), 2 (definite ATA), 3 (pronounced ATA) and 4 (loss of righting reflex).

Rats with a score of less than 2 were defined non ataxic.

Blood, brain and cerebellum of each animal were taken immediately after MES and stored as described previously.

Assessment of neurotoxicity

Animals were trained to remain on a rotarod apparatus (Dunham and Miya, 1957), set at 10 r.p.m., for a period of at least 60 sec. The training session was repeated 3 times (30 min apart) and animals showing inability to remain on the rotarod were discarded.

Four dose groups of 10 selected rats each were treated intraperitoneally with 12.5, 25, 50 and 100 mg/kg of denzimol suspended in 10 ml/kg of 0.25 % CMC.

The animals were retested 2 hr after administration of denzimol and the inability to remain on the rotarod for 60 sec was taken as evidence of impairment of coordinated motor function. None of the 20 untreated animals used as controls showed signs of neurotoxicity.

Immediately after testing, blood and brain of each animal were taken and stored as described previously.

Determination of denzimol in tissues

Tissue levels of denzimol were evaluated according to the method of Abbiati *et al.* (1985).

The tissues were deproteinizated in a mixture of 75 % acetone and 15 % 0.5N HCl. After addition of the internal standard 4,β-phenethyl-α-(2,4-dioxo-3-imidazolymidil)acetophenone the samples were alkalinized to pH 10 with NaOH and extracted with methylene chloride. Determination of denzimol was performed by HPLC (Waters) with a 25 cm Bondapack C18 column eluted with acetonitrile-0.03M KH_2PO_4 (50:50, v/v). Elution was monitored by UV absorbance at 214 nm using a Waters mod. 441 detector.

Calibration curves were obtained for plasma, brain and cerebellum after treating, as described above, homogenates of control tissues spiked with known concentrations of denzimol and internal standard.

The limit of detection of this method was of 250 ng per ml or per g.

Calculations

A two-compartment model with absorption phase was used for kinetic analysis of the plasma and tissues concentration versus time profile of denzimol.

Kinetic parameters were calculated using a Wang 2200 computer by the non linear fitting program described by von Hattingberg *et al.* (1977) with minor modifications.

The areas under the plasma or tissues concentration time curve (AUC) were calculated by the trapezoidal rule and extrapolated to infinite by adding Cn/β where Cn is the last value of the denzimol concentration in plasma or tissues over the calculated constant of elimination.

Linear regression analysis were performed on a Hewlett Packard 85 computer with standard programme.

Results

Time course of plasma, brain and cerebellum concentration of denzimol and its anticonvulsant effect against MES is reported in Table I. Maximum protection was observed as early as at 15 min after p.o. administration of 30 mg/kg and lasted for 8 hr.

Denzimol reached a peak at 15 min both in plasma and cerebellum, while in brain the tissue concentrations reached a plateau at 15 min and remained constant until 60 min after administration.

Figure 1 shows the semilogaritmic plot of the tissue concentrations of denzimol as a function of time. It appears that the disappearance of denzimol from brain and cerebellum follows a biphasic decay, with an initial phase lasting about 30 min, followed by a slower decline with an apparent half-life of 7–9 hr. In plasma, the decay of concentrations of denzimol in time was more irregular, and a second peak concentration was apparent after 6 hr, presumably due to enterohepatic recirculation of the product. For this reason, the tissue/plasma concentration ratio was not constant (see Table I).

The areas under the tissue concentration-time curves were 887, 9194 and 7414 $\mu g \times min \times ml^{-1}$ for plasma, brain and cerebellum, respectively.

The relationship between the concentration of denzimol in plasma, brain and cerebellum and its anticonvulsant and neurotoxic activities was examined in rats treated orally with different doses of the drug. The

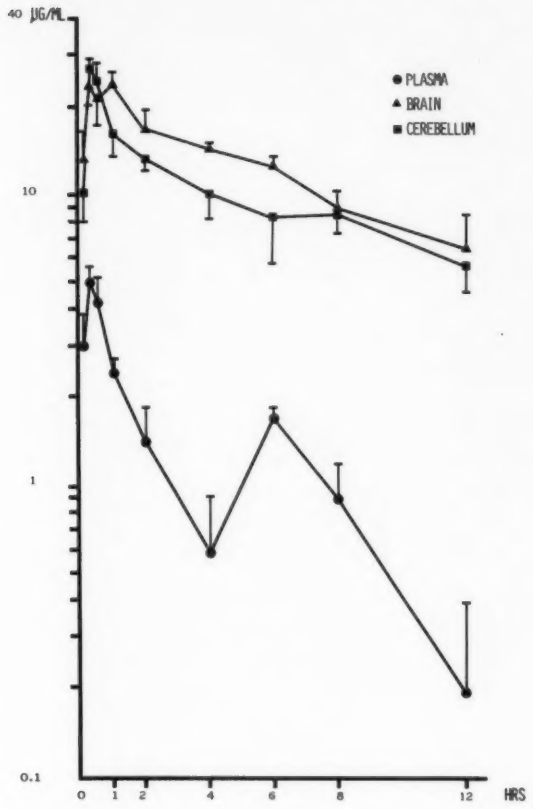


FIG. 1

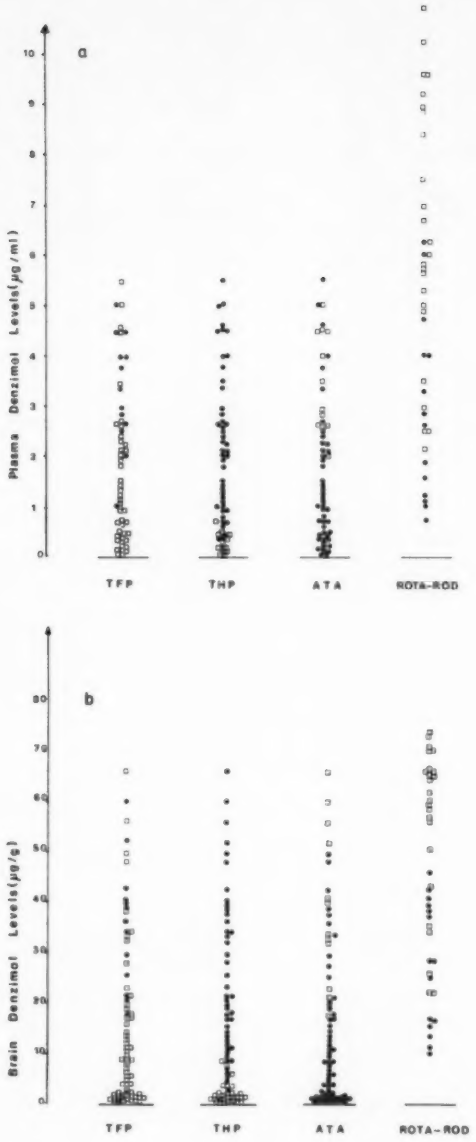
Plasma (●), brain (▲) and cerebellum (■) levels as a function of time after a 30 mg/kg single oral administration of denzimol. Each point represents the mean of five animals and vertical lines show the SEM.

animals were tested after 1 hr for presence or absence of ATA (visual scoring) and for protection against MES-induced seizures.

These quantal responses were plotted against the corresponding plasma levels of denzimol determined immediately after the test procedure.

The results for the three tissues examined are shown in Fig. 2.

Tonic hindlimb extension was always blocked when the plasma drug level exceeded 0.8 µg/ml. The forelimb extensor component was much more resistant to the effect of the drug than the hindlimb component. The


a
Plasma Denzimol Levels(µg/ml)
0
1
2
3
4
5
6
7
8
9
10
TFP
THP
ATA
ROTA-ROD
b
Brain Denzimol Levels(µg/g)
0
10
20
30
40
50
60
70
80
TFP
THP
ATA
ROTA-ROD

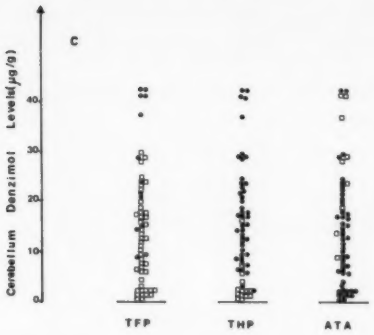


FIG. 2

Relationship between levels of denzimol and its anticonvulsant activity or neurotoxicity. Anticonvulsant activity was assessed against tonic fore and hind paws seizures (TFP-THP) induced by MES 1 hr after p.o. administration of different doses of denzimol. Visual observation of ataxia (ATA) was also performed on the same animals.

Rotarod performance was assessed 2 hr after i.p. administration of different doses of the drug.

The symbols indicate presence (□) or absence (●) of the symptoms, and each circle represents a single animal.

a = plasma; b = brain; c = cerebellum.

TABLE I

Plasma, brain and cerebellum concentrations of denzimol in rats (μg/ml or μg/g ± SEM) after oral administration of 30 mg/kg. Relationship between drug levels and anticonvulsant activity

Time after admin. (min)	Tissue*					
	Plasma	Brain	T/P	Cerebellum	T/P	THP**
5	3.05 ± 0.88	13.31 ± 3.21	4.36	10.20 ± 2.19	3.34	80
15	5.01 ± 0.73	23.65 ± 3.45	4.72	27.35 ± 1.69	5.46	100
30	4.31 ± 0.79	21.63 ± 4.03	5.02	24.79 ± 3.57	5.75	100
60	2.46 ± 0.25	23.93 ± 2.58	9.73	16.27 ± 2.64	6.61	100
120	1.42 ± 0.45	16.98 ± 2.53	11.96	13.52 ± 1.39	9.52	100
240	0.60 ± 0.33	14.48 ± 0.56	24.13	10.19 ± 1.89	16.98	100
360	1.72 ± 0.14	12.72 ± 1.28	7.39	8.49 ± 2.61	4.94	100
480	0.91 ± 0.29	9.17 ± 1.28	10.08	8.64 ± 1.25	9.49	100
720	0.20 ± 0.20	6.60 ± 2.07	33	5.77 ± 1.07	28.85	40

*Mean tissue levels of 5 animals.

**Percentage protection against tonic hindpaws extension induced by MES (10 animals).

T/P: Tissue/Plasma concentration ratio.

minimum effective level capable of eliciting a response was 1 μg/ml, but there was lack of complete abolition of convulsions even up to 5.5 μg/ml of denzimol.

The relationship between visually-assessed neurotoxicity and plasma concentration of denzimol reported in Fig. 2a, shows that in the range between 2 and 5.5 μg/ml some rats were scored as ataxic whereas others showed no neurotoxic symptoms. All rats with a plasma drug level equal or lower than 2 μg/ml displayed a complete lack of neurotoxicity.

In brain and cerebellum (Fig. 2b and c) a similar pattern was found: THP was always blocked at denzimol concentrations greater than 9 and 5 μg/g, respectively.

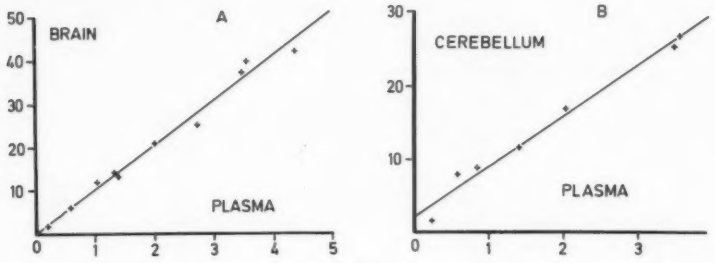


FIG. 3

Relationship between plasma levels and brain levels (A) or cerebellum levels (B) of denzimol. Regression analyses for brain and plasma levels give the equation $y = -0.24 + 10.37 \times (r = 0.991\ P < 0.01)$ and for cerebellum-plasma levels $y = 2.24 + 6.86 \times (r = 0.991\ P < 0.01)$.

Each point is the mean of 10 rats.

Ordinate: brain or cerebellum levels (μg/g tissue).

Abscissa: plasma levels (μg/ml).

None of the rats displayed ataxia at levels of the drug lower than 16 (brain) and 8.5 (cerebellum) μg/g, whereas ataxia was present only in some, but not in all, ofthe rats in the range level of 20–50 μg/g in both tissues.

In these animals, a linear relationship between drug concentrations in plasma, brain and cerebellum was obtained by microcomputer analysis in the range of the doses administered (1 to 128 mg/kg), as shown in Fig. 3. Brain/plasma ratio was calculated to be 10.37 whereas cerebellum/plasma ratio was about 7. The data points in the figures represent the mean plasma, brain and cerebellum concentrations measured in the various dose groups used in the MES procedure.

One of the main objectives of this study was to establish an effective tissue concentration range for denzimol in experimental epilepsy.

Therefore, emphasis was placed on the determination of plasma levels of the drug that could be associated with clear neurological damage.

After oral administration of denzimol up to 128 mg/kg, no clear induction of ataxia was observed.

Different doses of the drug were then administered intraperitoneally. The rotarod performance of each animal dosed was evaluated and plasma and brain levels of denzimol were determined.

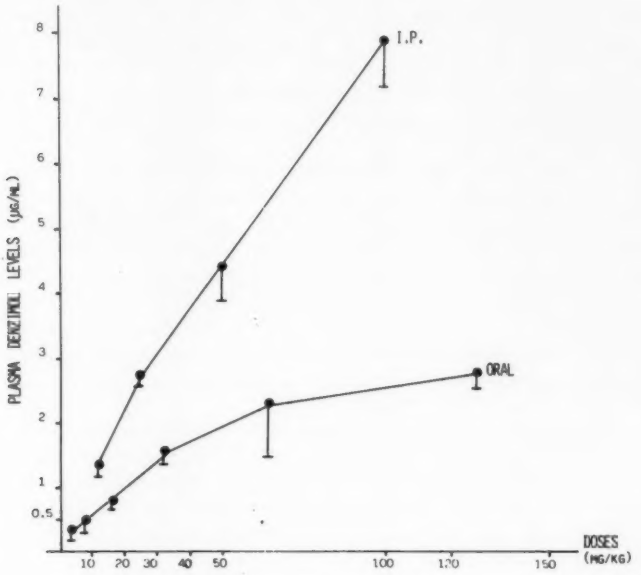


FIG. 4

Mean plasma levels of denzimol, 2 hr after intraperitoneal or oral administration of different doses of the drug. Each point is the mean of 5–10 rats and vertical lines show the SEM.

Indeed Fig. 4 shows that after intraperitoneal administration, plasma levels of denzimol are higher than the levels obtained after an oral dosing of the drug.

The relationship between tissue concentrations and rotarod performance for plasma and brain levels of the drug is reported respectively in Fig. 2a and 2b.

Visually-assessed neurotoxicity and rotarod performance had a good degree of correlation.

At plasma levels of the drug lower than 2 µg/ml no rat showed ataxia or

rotarod impairment, whereas a complete motor disturbance was observed at plasma levels of denzimol higher than 5.6 μg/ml. This level was never reached after oral administration.

A similar finding was made in brain (Fig. 2b).

Discussion

It has been reported by Masuda *et al.* (1979) that in the rat the p.o. administration of 50 mg/kg of phenytoin significantly protects animals (>50 %) from MES induced seizures in a time-range of 2–6 hr after administration. Similarly, 20 mg/kg of phenobarbital and 50 mg/kg of carbamazepine show a time-range of protection of 1–8 and 1–4 hr, respectively.

Our present results show that denzimol, administered to rats at 30 mg/kg shows an anticonvulsant activity lasting at least 8 hr.

Maximum protection was observed already at 15 min after administration of the drug, whereas with the reference anticonvulsants, the maximum protection was reached after 2 hr for phenytoin, 2 hr for phenobarbital and 1 hr for carbamazepine. Furthermore, it should be noticed that, while denzimol was given at a concentration equal to its ED_{50}, the reference drugs were administered in concentrations significantly higher than the respective ED_{50}, calculated by Swingard and Wolf (1981, personal communication) at peak time.

The results of this investigation demonstrate that plasma levels of denzimol of 0.8 μg/ml and above were always associated with complete abolition of tonic hindlimb extension induced by electroshock.

Moreover, there is a clear separation between the effective anticonvulsant plasma concentration of denzimol and the one which produced marked neurotoxicity (above 6.5 μg/ml).

When these results were calculated in terms of protective index (P.I. = neurotoxic level divided by anticonvulsant level), it was shown that denzimol (P.I. = 8.1) is more similar to AD-810 (P.I. = 8.8) than to phenytoin, carbamazepine, phenobarbital or valproate, whose protective indices were published to be, respectively: 5, 2.5, 2.4 and 2.2 (Masuda *et al.*, 1979; Tullock *et al.*, 1982).

As reported above, plasma levels of about 6–6.5 μg/ml are necessary to cause significant neurotoxic effect in the rat. The fact that after oral administration of doses of denzimol up to 128 mg/kg these plasma levels are never reached, explains the lower incidence of side effects induced by this drug.

Denzimol shows an excellent linear relationship between drug concentrations in plasma and those in brain both after oral or intraperitoneal administration.

In addition, the brain/plasma concentration ratio of this drug was, 1 hr after treatment, approximately 10 to 1, whereas for the other anticonvulsants (phenytoin, carbamazepine), this ratio is roughly equal to 1 (Masuda *et al.*, 1979).

Masuda *et al.* (1979) reported that the therapeutic ranges of plasma concentrations of phenytoin, phenobarbital and carbamazepine determined in various species of animals were comparable to those recommended clinically and that these concentrations determined in animal models of seizures were relatively constant irrispective of species and models utilized.

Taking into account these considerations, the results obtained in the present study seem to suggest that denzimol is effective at brain concentrations higher than 8 μg/g, with a therapeutic range up to 60 μg/g, which is much wider than the effective ranges of phenytoin, phenobarbitone, carbamazepine. Moreover, denzimol appears to be effective at 10 fold lower plasma levels that the other reference anticonvulsant drugs.

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Effects of Manganese Ions on Verapamil-Induced Increase of Spontaneous Transmitter Release in Motor Nerve Endings of Rat Diaphragm Preparations

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Abstract—Effect of verapamil on the spontaneous transmitter release in the presence and absence of external calcium and manganese ions was examined by monitoring the frequency of miniature end-plate potentials (MEPPs) in the rat diaphragms. Verapamil (25 to 50 μM) caused a sustained increase in the MEPP frequency in a normal Ringer solution and a transient increase in a calcium-free solution without change in the resting potential of end-plate. A readmission of calcium restored the sustained stimulatory effect of verapamil. The stimulatory effect of verapamil in calcium-free solution and the restoration of the effect by the calcium readmission were both blocked by pretreatment with manganese ions. It is suggested that verapamil increases the spontaneous transmitter release from the motor nerve terminal by increasing axoplasmic calcium levels through release from intracellular stores. Calcium may be continuously supplied to these stores from the extracellular space in the presence of verapamil through a pathway that is inhibited by manganese.

Introduction

Verapamil, an organic calcium antagonist, inhibits calcium entry into squid giant axon (Baker, 1972; Baker *et al.*, 1973a and b), cardiac muscle

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(Kohlhardt *et al.*, 1972), vascular (Golenhofen and Hermstein, 1975) and intestinal (Riemer *et al.*, 1974) smooth muscle, and thereby reduces intracellular calcium-mediated processes. Though it is necessary to increase calcium ion concentration in the motor nerve terminal for evoked transmitter release (Katz and Miledi, 1967), it has been reported that verapamil does not inhibit the twitch response in the mouse diaphragm muscle after phrenic nerve stimulation, but rather increases selectively the twitch tension (Asai *et al.*, 1981 and 1982b). Moreover, verapamil increased the transmitter release at the neuromuscular junction of the frog (Publicover and Duncan, 1979; Gotgilf and Magazanik, 1977) and rat (Asai *et al.*, 1982a; Nishimura *et al.*, 1982). Furthermore, in rats, verapamil has no inhibitory effect on potassium-stimulated transmitter release from the motor nerve terminal (Asai *et al.*, 1982a). Based on these reports, it has been assumed that verapamil increases the spontaneous transmitter release by elevating axoplasmic calcium levels. Since verapamil is transiently effective in calcium-free solutions (Nishimura *et al.*, 1982), the excess calcium is probably released from intracellular stores. The mechanism, however, which supports the sustained effect of verapamil in normal solution is uncertain.

On the other hand, the spontaneous transmitter release at the neuromuscular junction is partly dependent on external calcium (Elmqvist and Feldman, 1965), thus it can be partially regulated in the resting membrane by external calcium. Manganese ion antagonizes the effect of calcium and inhibits potassium-stimulated transmitter release at the rat neuromuscular junction (Asai *et al.*, 1982a).

Present experiments have been carried out to elucidate the effects of calcium readmission and manganese treatment on verapamil-induced increase in spontaneous transmitter release in motor nerve endings of rat diaphragms.

Methods

Left hemidiaphragms of male Wistar rats, weighing 200 to 250 g, were placed in a 20 ml organ bath containing Krebs-Ringer solution (136 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM $NaHCO_3$, and 11 mM glucose). The experiments in calcium-free buffer were performed in Krebs-Ringer solution without calcium chloride. The solution was bubbled with a mixture of 5 % CO_2 and 95 % O_2, and kept at pH 7.3 and a temperature of 37° C.

Transmembrane potentials of individual muscle fibers were measured by conventional intracellular techniques. Glass micro-electrodes with tip resistance of 6 to 10 MΩ, when filled with 3 M KCl, were used. They were

inserted into fibers near end-plate regions. The electrode was connected to a high input impedance amplifier (Nihon Kohden, MEZ-8201), and the output of this amplifier was fed to an oscilloscope (Nihon Kohden, VC-9), and stored on magnetic tape (Nihon Kohden, RMG-5204). Spontaneous MEPPs were analyzed by a computer (Nihon Kohden, ATAC-350 or DAB-1100). MEPPs were recorded for every successive 1 min after exposure to a given solution. The mean MEPP frequency (number per sec) was calculated for each end-plate from these recordings. The experiments were performed only when the MEPP frequency was stable in control solution.

Drugs used: verapamil (donated by Eisai), manganese chloride (Wako Pure Chemicals). All other chemicals were of analytical grade.

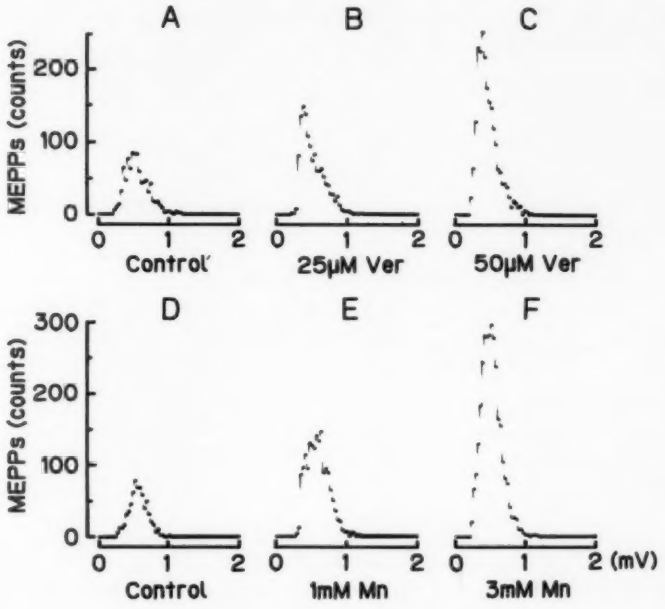

FIG. 1

Amplitude-histograms of miniature end-plate potentials (MEPPs) at the rat neuromuscular junctions in standard Krebs-Ringer solution containing verapamil (Ver, 25 and 50 μM) and manganese ions (Mn, 1 and 3 mM). Histograms A, B and C and histograms D, E and F were taken from the same end-plate, resp. MEPPs in B, C, E and F were recorded from 10 to 15 min after addition of each agent. MEPPs for successive 5 min were estimated at 920, 1348, 2202, 564, 1681, and 3494 counts for A, B, C, D, E, and F, resp.

Results

Fig. 1 shows amplitude-histograms of MEPPs recorded from control (A and D), verapamil- (B and C) and manganese-treated (E and F) tissue. Each histogram represents successive 5 min counts of MEPPs. Verapamil (25 and 50 μM) elevated the frequency of MEPPs, indicating an increase in the spontaneous transmitter release. The stimulatory effect was reversible, reproducible and concentration dependent. Ten to 15 min after its addition, verapamil (50 μM) increased the average frequency by a factor of three (Table I). This effect lasted at least 40 min. Verapamil (25 and 50 μM) did not alter the resting potential of the end-plate.

Manganese ions (1 and 3 mM) also increased the frequency of MEPPs. The stimulatory effect was reversible, reproducible and concentration dependent. Ten min after the application, manganese ions (3 mM) raised the average frequency by a factor of four (Table I).

TABLE I

Modification by external calcium of effects of verapamil (Ver, 50 μM) and manganese ions (Mn, 3 mM) on the frequency of miniature end-plate potentials at the rat neuromuscular junction

	Frequency (sec^{-1})		
	Standard K-R	Ca-free K-R	Ca-readmission
Control	2.8 ± 0.48 (24)	0.9 ± 0.33 (31)	$2.7 \pm 0.50^{b**}$ (28)
Ver	$7.8 \pm 1.96^{a**}$ (16)	$2.9 \pm 0.43^{a**}$ (10)	$7.5 \pm 1.8^{b*}$ (13)
Mn	$11.5 \pm 2.80^{a***}$ (12)	$3.3 \pm 0.50^{a***}$ (10)	3.0 ± 0.78 (12)

Control values in calcium-free Krebs-Ringer (Ca-free K-R) solution were obtained 30 min or more after calcium-removal. Ver or Mn was added approximately 40 min after the calcium-removal; their effects were determined in some end-plates from 10 to 15 min after their application. Ca-readmission: 2 mM calcium was added approximately 70 min after the calcium-removal; miniature end-plate potentials were determined in some end-plates from 10 to 15 min after the Ca-readmission. Values are mean $\pm$ s.e.m. Number of end-plates are presented in the parenthesis. ^{a**} and ^{a***}: control vs Ver or Mn, $P < 0.05$ and $P < 0.01$, resp. ^{b*} and ^{b***}: Ca-free K-R vs Ca-readmission, $P < 0.05$ and $P < 0.01$. resp.

When calcium was removed from the Krebs-Ringer solution the frequency of MEPPs gradually decreased, in 30 min to about one third both in the controls and in the presence of verapamil or manganese (Table I). The readmission of 2 mM calcium gradually restored the MEPP frequency to the control level in approximately 10 min in the controls and in the presence of verapamil, but not in the presence of manganese.

Verapamil (50 μM) applied 40 min after the calcium-removal increased the frequency of MEPPs. This stimulatory effect disappeared within 20 min after its application (data not shown). A readmission of 2 mM calcium 10 min after the disappearance of the effect of 50 μM verapamil restored and sustained the effect (Table I). Manganese ions also elevated the MEPP frequency in calcium-free solution (Table I). A readmission of 2 mM calcium 30 min after the manganese treatment, however, did not show any further increase in the MEPP frequency, Moreover, 1 mM manganese blocked the stimulatory effect of verapamil in calcium-free solution and the sustained effect of verapamil observed after the readmission of 2 mM calcium (Fig. 2).

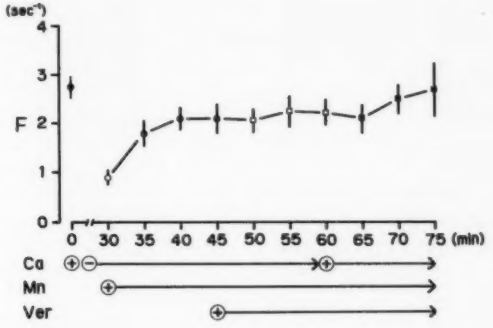

FIG. 2

Lack of effects of 50 μM verapamil (Ver) and of readmission of calcium (2 mM) on the frequency of miniature end-plate potentials (MEPPs) at the rat neuromuscular junctions in calcium-free solution containing 1 mM manganese ions (Mn). The frequency (F) of MEPPs are shown as number per second. Results are expressed as mean $\pm$ s.e.m. from separate 8 experiments.

Discussion

In the present experiments verapamil and manganese elevated MEPP frequency at the rat neuromuscular junction, indicating an increase in spontaneous transmitter release. This observation is consistent with previous findings on the spontaneous quantal release caused by verapamil on the frog (Gotgilf and Magazanik, 1977; Publicover and Duncan, 1979) and rat (Nishimura *et al.*, 1982; Asai *et al.*, 1982a) and by manganese on the frog neuromuscular junction (Meiri and Rahamimoff, 1972). It has been suggested that the presynaptic stimulatory effect of verapamil (Publicover and Duncan, 1979; Nishimura *et al.*, 1982) and manganese (Balnabe and Gage, 1973) are due to an increase in the concentration of

cytoplasmic calcium. As shown here, both agents are effective in calcium-free solution, suggesting that their effects are due to the release of calcium from intracellular stores. The stimulatory effect of verapamil in calcium-free solution was masked in the presence of manganese ions. This may imply that verapamil and manganese act competitively on similar calcium stores in the nerve terminal.

The effect of verapamil in calcium-free buffer was found to be transient in a previous (Nishimura *et al.*, 1982) and in the present study. A readmission of calcium restored the sustained stimulatory effect of verapamil. This effect of verapamil was blocked by manganese ions. Manganese ions also inhibit potassium-stimulated transmitter release at the rat neuromuscular junction (Asai *et al.*, 1982a). These data suggest that the sustained effect of verapamil requires extracellular calcium ions. This indicates that it is probably dependent upon the influx of calcium through channels that can be blocked by manganese ions. Since the calcium readmission failed to facilitate MEPP frequency in the presence of manganese ions, such channels are probably effective to regulate, at least partially, the spontaneous transmitter release in the resting membrane by external calcium.

Theoretically verapamil may increase the cytoplasmic calcium concentration by facilitating the transmembrane influx of extracellular calcium or by increasing the release of calcium from intracellular stores. It is also conceivable, however, that verapamil promotes the utilization of the cytoplasmic calcium by the transmitter release mechanism. The finding that verapamil had no effect on the membrane potential of the nonstimulated (resting) end-plate makes it unlikely that it facilitates the influx of calcium into the cytoplasm. It is more likely that the increase of the cytoplasmic calcium concentration caused by verapamil is due to the facilitation of the release of calcium from intracellular stores. The sustained increase of MEPP frequency caused by verapamil requires the replenishment of the intracellular calcium stores by influx of extracellular calcium. This influx probably occurs through channels that can be, at least partially, blocked by manganese.

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Effect of Stress and Glucocorticoids on the Gastrointestinal Cholinergic Enzymes

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Abstract—Male Sprague-Dawley rats maintained under controlled lighting and temperature conditions were used in this experiment. Animals were exposed to acute cold (4° C) and immobilization stress for one hour, exposed to cold stress for 7 days (chronic stress) or treated with corticosterone (2 mg/kg) 1 hr prior to sacrificing. Animals with their proper controls were sacrificed and the stomach, duodenum, ileum and colon were separated and assayed for choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities. The data obtained indicated that exposure to acute stress resulted in significant decline in ChAT activity in all tissues studied. The administration of corticosterone resulted in significant decline in ChAT activity in all tissues studied except for the duodenum. Meanwhile, the exposure to chronic stress did not have any significant effect on ChAT activity. On the other hand, acute stress caused significant increase in AChE activity in all tissues studied except for the ileum and stomach. The duodenal AChE activity of stressed animals increased thirty-fold when compared to control. The administration of glucocorticoids significantly reduced AChE in all tissues studied, except for the duodenum and stomach where there was thirty-two-fold increase as compared to the control levels. The exposure to chronic stress also caused significant increase in AChE of all tissues studied, except for the colon. The results of this experiments indicate that the duodenal AChE is extremely sensitive to stress or glucocorticoids and that stress induced changes in the cholinergic enzymes of the gastrointestinal tract may be mediated by adrenal steroids.

Introduction

Cholinergic activity exerts a considerable influence on the gastrointestinal function (Bunce *et al.*, 1977). These cholinergic influences are controlled by long vago-vagal reflexes (Debas *et al.*, 1975) and by local cholinergic reflexes which have been identified in the absence of vagal innervation (Magee and Hu, 1975). Ulcer formation has been linked to acid secretion (Anderson, 1967). Gastric emptying and acid secretion were found to be controlled by cholinergic receptors in the duodenum (Hunt and Knox, 1968). A possible role for a local cholinergic mechanism in the control of acid secretion has been studied using atropine in animals in which the vagal innervation to stomach or its fundic pouch has been severed (Hunt and Knox, 1968). Vizi *et al.* (1972, 1973) have demonstrated that gastrin can release acetylcholine from the myenteric plexus in the guinea pig ileum. They also suggest that the secretagogue action of gastrin in the stomach is partly mediated through the release of acetylcholine.

Recent results from this laboratory have shown that there is an inhibition in AChE activity in rat hypothalamus and the adrenal gland when animals were exposed to acute or chronic stress or after the administration of corticosterone (Gabriel and Soliman, 1983). These effects might be due to the decreased synthesis and/or increased degradation of the enzyme. It was also observed that the administration of corticosterone to intact animals caused the same effect on AChE as in the case of stress exposure which indicates that stress-induced decline of AChE might be mediated through the adrenal cortex (Gabriel and Soliman, 1983).

For many years, investigators have examined the pathogenesis of stress-induced ulcers. In spite of numerous studies and hypothesis, it appears that no single etiological mechanism can be pinpointed as the causative factor for the development of these ulcers. At present these ulcers are viewed as a multifactorial phenomenom. One of the factors studied extensively has been the role of the adrenal gland. Weiss (1971) had demonstrated that rats that showed very high steroid levels also showed very severe ulcerations. Meanwhile, Murphy *et al.* (1979) showed that in stressful situations, animals with hippocampal lesions have enhanced adrenocortical activity with an increase in the incidence of gastric ulcers. On the other hand, stress and glucocorticoids have been implicated in different gastrointestinal diseases. Emotional disturbance and physical stress have been linked to diarrhea (Sleisenger and Fordtran, 1978) and to irritable bowel syndrome (Almy, 1976).

Therefore, the present investigation was designed to study the effect of stress or glucocorticoid on the acetylcholine synthesizing enzyme (choline acetyltransferase, ChAT) and acetylcholinesterase, (AChE) in different regions of the gastrointestinal tract.

Methods

Male Sprague-Dawley rats weighing 130–160 g and purchased from Southern Animals Farms (Prattville, AL) were used in this study. They were adapted to a temperature of $21 \pm 1°$ C in an environmental chamber equipped to provide 23.25 cd/m^2 of cool, white fluorescent light for a minimum period of one week. The light period in the chamber was automatically timed to last from 08.00 to 20.00 hr followed by a 12-hour dark period. Standard pellet diet (Purina, St. Louis, MO) and water were provided ad libitum.

In this experiment, 24 rats were randomly divided into 4 groups. In the first group, animals were exposed to cold stress at 4° C of ambient temperature; they were also simultaneously immobilized for 1 hr (acute stress group). The second group was subjected to the same cold stress (without immobilization) for 7 days (chronic stress group). Another group of animals were injected with corticosterone (Sigma Chemical Company St. Louis, MO; 2 mg/kg, i.p.) 1 hr prior to sacrificing. Six other animals were kept under normal environmental conditions and were used as a control group.

All the animals were sacrificed by decapitation at 10.00 hr. The stomach, duodenum, ileum and colon were rapidly separated on ice and the mucosa of each region was gently scrapped by means of a scalpel. AChE was assayed using the procedure of Ellman *et al.* (1961). Each tissue was individually homogenized (1 % W/V) in 0.1 M ice-cold phosphate buffer (pH 7.4) containing 0.1 % Triton X-100 (Sigma). AChE activity in each homogenate was immediately determined by the spectrophotometric method and expressed as micromoles of substrate (acetylthiocholine iodide; Sigma) hydrolyzed per minute per gram of tissue.

For ChAT activity each tissue was individually homogenized (1 % W/V) in 0.1 M ice-cold phosphate buffer (pH 7.0). ChAT activity was determined using the spectrophotometric method of Chao and Wolfgram (1972). ChAT activity was expressed as nanomoles of COASH formed per minute per gram of tissue.

All data were analyzed using a Hewlett-Packard 9815A Computer. Overall comparisons between the different groups were performed by one-way analysis of variance (Steel and Torrie, 1960). The significance of the difference between the means of various tissues was determined using the LSD test (Steel and Torrie, 1960).

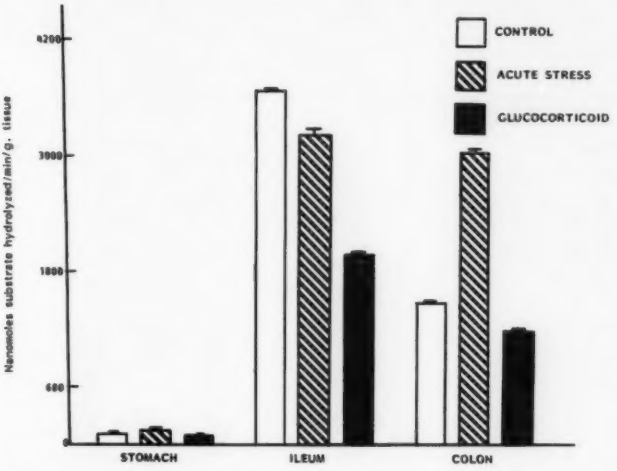

FIG. 1

Effect of immobilization and cold stress (acute stress) or glucocorticoid (2 mg/kg) treatment on the activity of AChE in the stomach, ileum and colon. Each bar represents the mean of 6 animals ± SEM.

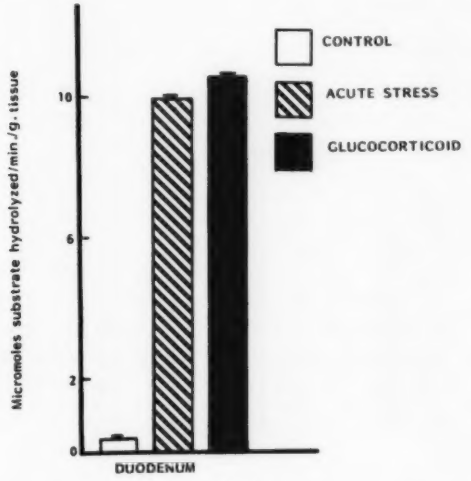

FIG. 2

Effect of immobilization and cold stress (acute stress) or glucocorticoid (2 mg/kg) treatment on the activity of AChE in the duodenum. Each bar represents the mean of 6 animals ± SEM.

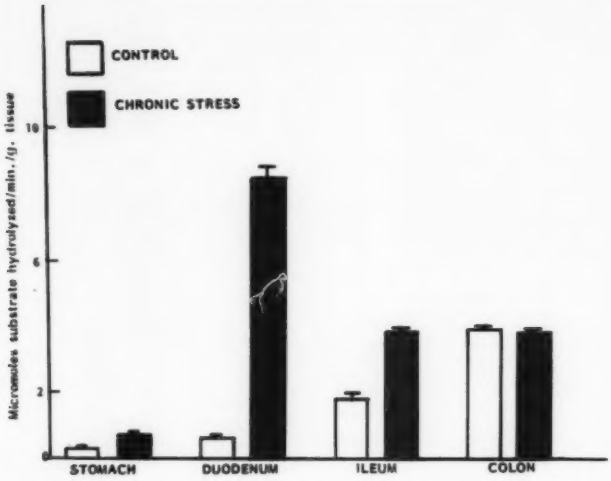

FIG. 3

Effect of cold exposure for 7 days at 4°C (chronic stress) on the activity of AChE in the stomach, duodenum, ileum and colon. Each bar represents the mean of 6 animals ± SEM.

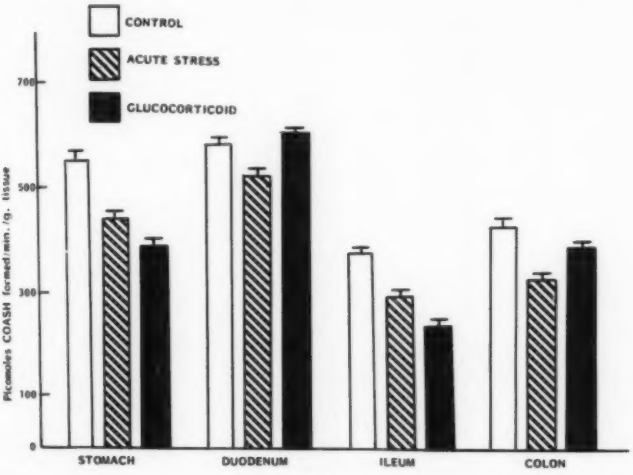

FIG. 4

Effect of immobilization and cold stress (acute stress) or glucocorticoid (2 mg/kg) treatment on the activity of ChAT in the stomach, duodenum, ileum and colon. Each bar represents the mean of 6 animals ± SEM.

Results

Figures 1 and 2 show the effect of acute stress or glucocorticoid treatment on the AChE activity in different regions of the gastrointestinal tract. In comparison with untreated controls, acute stress caused a significant increase ($p < 0.001$) in enzyme activity in the duodenum (3145 %), colon (75 %), and a significant decrease ($p < 0.001$) in enzyme activity in the duodenum (3334 %) and a significant decrease ($p < 0.01$) in enzyme activity in the ileum (44.3 %), and colon (33.0 %) as shown in Figs. 1 and 2. There was no significant change in AChE activity in the stomach ($p > 0.05$).

Figure 3 depicts the activity of AChE in chronic stress animals compared to the control group. The results show significant increase ($p < 0.05$) in enzyme activity in the stomach (54.5 %), duodenum (1627 %), ileum (121 %) and no significant change in the colon.

Figure 4 represents the activity of ChAT in the different gastrointestinal tract regions studied in control, acute stress rats and corticosterone treated animals (2 mg/kg i.p.). The results show that there was significant decline ($p < 0.05$) in the stomach (19.1 %), duodenum (10.3 %), colon (24.7 %), and ileum (21.0 %) of the acute stress groups. In comparison with the control group, corticosterone treatment resulted in a significant decrease ($p < 0.05$) of ChAT activity in the stomach (29.1 %), colon (9.41 %) and ileum (35.1 %) with no significant effect in the duodenum.

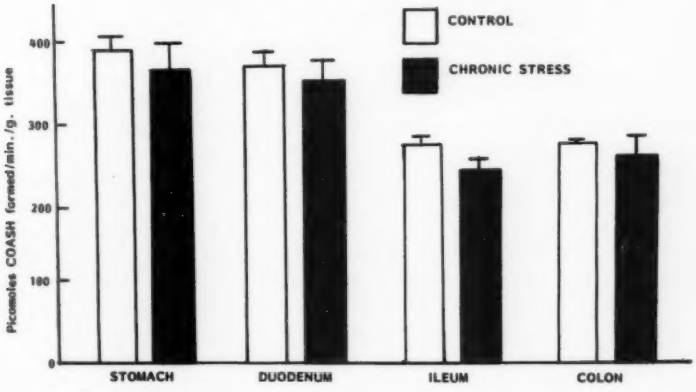

FIG. 5

Effect of cold exposure for 7 days at 4° C (chronic stress) on the activity of ChAT in the stomach, duodenum, ileum and colon. Each bar represents the mean of 6 animals ± SEM.

Figure 5 summarized ChAT activity in the different regions studied in control and chronic stress animals. There was no significant change in ChAT activity in all tissues studied.

Discussion

The results of the study indicate that the activity of AChE and ChAT varies in different regions of the gastrointestinal tract, and that the duodenal AChE is extremely sensitive to stress or glucocorticoid administration. The current findings also suggest that stress-induced changes in the cholinergic enzymes of the gastrointestinal tract may be mediated by adrenal steroids.

The fact that administration of corticosterone to intact rats caused the same effect as that seen in acute stress animals, gives support to the fact that stress-induced changes in AChE may be mediated by adrenal steroids. Although we have not measured the levels of corticosterone in the plasma, it has been reported that following acute or chronic exposure to cold stress, a rise in the circulating levels of corticosterone was noted in the rat (Sakellaris and Vernikos-Danellis, 1975). Tache' *et al.* (1976) noted that female rats exposed for 3 days of stress showed an inhibition in body weight and an induced adrenal enlargement. These authors also showed that after six days of stress there was stabilization in the adrenal weight. An increase was also noted in the plasma levels following 150 minutes of daily immobilization (Mikulaj *et al.*, 1974).

Incidence of gastric and peptic ulcers have been related to stress (Murphy *et al.*, 1979). Some probable causes are hypersecretion of gastric acid and the development of ischemic areas in the gastroduodenal mucosa (Leonard *et al.*, 1964). Leonard *et al.* (1964) have postulated that any severe stress may cause prolonged ischemia of the gastroduodenal mucosa. They note that strong sympathetic stimulus and high circulating catecholamines caused by severe stress may open arteriovenous shunts in the gastroduodenal submucosa. Because of this phenonmenom, blood which normally flows through the capillary bed of the gastroduodenal mucosa, then flows instead through the submucosal arteriovenous shunts and is thus diverted from the gastroduodenal mucosa. As a result of this, the mucosa is severely injured. The ulceration develops when areas of mucosa undergo necrosis. Anticholinergic drugs have been used to treat these ulcers on the assumption that they shunt blood away from the bleeding mucosa by blocking excessive splanchnic parasympathetic activity and thus opening submucosal arteriovenous shunts (Leonard *et al.*, 1964).

Choline acetyltransferase activity has frequently been used as a reliable marker of the cholinergic nerve activity. In this study, biochemical measurements of ChAT and AChE following stress revealed changes in

cholinergic activity of the mucosa of gastrointestinal tract region studied. Similar results were obtained when animals were treated with corticosterone. The results obtained are an indication of the mucosal reaction when the body is exposed to stress. The decline in the cholinergic function of different regions of the gastrointestinal tract may explain the ischemia produced in gastroduodenal mucosa following stress (Leonard *et al.*, 1964). The results also indicate that adaptation can take place in the cholinergic activity when animals were exposed to chronic stress conditions. There was no observed change in ChAT activity in animals exposed for 7 days to cold stress.

It is interesting to note the rapidity of the activation of AChE in the duodenum. The dramatic increase in AChE is probably associated with a direct activating effect on the enzyme rather than an increase in the rate of enzyme synthesis in the gastrointestinal mucosa.

Although the outcome of this investigation was not expected, the present work provides very significant information regarding the role of stress and glucocorticoids in the regulation of the cholinergic enzymes of the gastrointestinal tract. Additional information could be obtained by assaying for the actual levels of acetylcholine in the gastrointestinal tract mucosa in relation to stress.

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Blocking Effects of Verapamil on Pancreatic Exocrine Secretion Induced by Dopamine in the Dog

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Abstract—The effects of verapamil on the dopamine-induced pancreatic exocrine secretion were investigated in the isolated and blood-perfused canine pancreas at a constant flow rate *in situ*. All drugs were given intra-arterially. Dose-related increases in the volume of pancreatic secretion induced by dopamine (1–10 μg) were reduced by infusion of 50 and 100 μg/min of verapamil, but were not affected by diltiazem (100 μg/min) and dilazep (100 μg/min) infusions. Protein concentration in pancreatic juice induced by dopamine was decreased significantly by the infusion of verapamil, diltiazem and dilazep, but bicarbonate concentration was not. These results suggest that verapamil reduced the dopamine-induced pancreatic secretion by virtue of its dopamine antagonist activity and inhibited protein secretion by virtue of its calcium channel blocking action.

Introduction

Verapamil is used clinically in the treatment of angina pectoris (Melville *et al.*, 1964; Schamroth, 1971) and supraventricular arrhythmias (Schamroth *et al.*, 1972; Rosen *et al.*, 1975). The action of verapamil seems to inhibit the slow calcium-dependent ionic channels of depolarizing cells (Fleckenstein *et al.*, 1968; Kohlhardt *et al.*, 1972). In addition to blocking the entry of calcium into the cells, verapamil inhibits radioligand binding to adrenergic and muscarinic receptors in rat myocardium (Glossman and Hornung, 1980; Karliner *et al.*, 1982) and to adrenergic receptors in human platelets, rat kidney and heart (Motulsky *et al.*, 1983). Recently, it was reported that verapamil displaced spiperone, dopamine antagonist, in

membrane preparations of the porcine anterior pituitary (Cronin, 1982) and antagonized the action of dopamine on the isolated perfused ear artery (Johnson *et al.*, 1983). The authors suggested that antagonist effect of verapamil should be evaluated on other tissues in which dopamine receptors exist. As the secretagogue effect of dopamine was antagonized by haloperidol (Furuta *et al.*, 1973) and sulpiride (Honda *et al.*, 1977; Iwatsuki *et al.*, 1983), there are specific dopamine receptors related to the exocrine secretion in the dog pancreas. Previously we reported that a small dose of verapamil caused a slight and not significant inhibition of the secretory response to dopamine in dogs (Iwatsuki *et al.*, 1982b).

In the present study, we have examined the effects of a large dose of either verapamil or other calcium channel blockers, diltiazem or dilazep, on pancreatic exocrine secretion induced by dopamine and secretin in the isolated and blood-perfused canine pancreas *in situ*.

Methods

Experiments were performed on 12 ± 0.5 kg (mean $\pm$ S.E., $n = 28$) mongrel dogs of either sex that were maintained for 24 hr without food but were allowed free access to water. The animals were anesthetized with sodium pentobarbital (30 mg/kg, i.v.) and tied in a supine position. A cuffed endotracheal tube was inserted and animals were ventilated with room air. A catheter was inserted into the right femoral vein for the administration of additional doses of pentobarbital and infusion of saline. Systemic arterial blood pressure was monitored with an electromanometer (Nihon Kohden RP-3) through a catheter inserted into the right femoral artery. The abdomen was opened, and the duodenum exposed. A longitudinal incision was made in the duodenal wall, and a polyethylene catheter (inside diameter, 0.8 mm; outside diameter, 1.3 mm) inserted through the papilla into the main pancreatic duct. The catheter was secured to the duodenum, and the drops of pancreatic juice flowing out of the catheter were counted with a drop counter. The accessory pancreatic duct was ligated. The pancreas was hemodynamically isolated *in situ* and perfused at a constant blood flow rate according to the procedure described and illustrated diagrammatically by Hashimoto *et al.* (1971) and Iwatsuki *et al.* (1982a). The right gastric artery and the right gastroepiploic artery arising from the gastroduodenal artery were ligated. The gastric branches of the splenic artery were also ligated, and a catheter was inserted into the gastroduodenal artery. After splenectomy another catheter was inserted retrogradely into the splenic artery. Both these catheters in the gastroduodenal and splenic arteries were connected to a Y-shaped tube, and the pancreas was perfused with blood withdrawn from the left femoral artery. The flow rate was measured and kept constant (15–20 ml/min).

After the extracorporeal circuit was established, the proximal portion of the splenic artery and the inferior pancreaticoduodenal artery were ligated. This procedure allowed isolation of the pancreas without any interruption of the blood supply during surgery. The incised abdomen was covered with saline-soaked gauze, and kept warm with an electric lamp. After completion of surgery, and before establishment of the perfusion circuit, the animals were given i.v. a dose of 500 units/kg of sodium heparin and then a maintenance dose of 100 units/kg/hr for the duration of the experiment. Dopamine and secretin were injected into a rubber tube connected to the shank of the arterial cannula leading to the pancreas, over a period of 2 sec, using a microinjector. After obtaining responses to these secretagogue drugs, verapamil, diltiazem or dilazep was infused at a constant rate using an infusion pump (Harvard Apparatus, model 600–900) and the effect of secretagogues was reassessed during the infusion. Pancreatic juice was collected for measurement of volume as well as concentrations of bicarbonate and protein. The concentration of bicarbonate in juice was measured by a blood gas analyzer (Corning, model 165/2) which requires 175 μl of minimal sample volume. Protein concentration in juice was measured by the method of Bradford (1976), using bovine serum albumin as the standard. The volume of juice, bicarbonate or protein concentration in juice is expressed as increase from that of the resting value. Drugs used in this study were dopamine hydrochloride (Sigma), secretin (Eisai, 1 mg = 1.5×10^5 CHR units), verapamil hydrochloride (Eisai), diltiazem hydrochloride (Tanabe) and dilazep (Kowa). Secretin was dissolved in 0.9 % saline solution to which bovine serum albumin had been added, to give a 0.1 % solution (Singer *et al.*, 1981); the other drugs were dissolved in 0.9 % saline. The dose of secretin is indicated in Crick, Harper and Raper units (Crick, Harper and Raper, 1950). Statistical analysis was carried out by means of the Student's *t*-test.

Results

Effects of Dopamine and Secretin on Pancreatic Exocrine Secretion and its Modification by Verapamil, Diltiazem and Dilazep

Administration of dopamine (1,3 and 10 μg) into the arterial cannula leading to the pancreas produced a dose-related increase in the secretion of pancreatic juice (Fig. 1A). Responses lasted not more than 10 min, as reported previously (Iwatsuki *et al.*, 1982b). Verapamil, infused i.a. at a rate of either 50 or 100 μg/min, did not induce any secretory effects. However, during an infusion of 50 μg/min of verapamil, dopamine-induced secretion was significantly reduced. The larger infusion dose of verapamil (100 μg/min) caused a more marked shift to the right of the dose-response

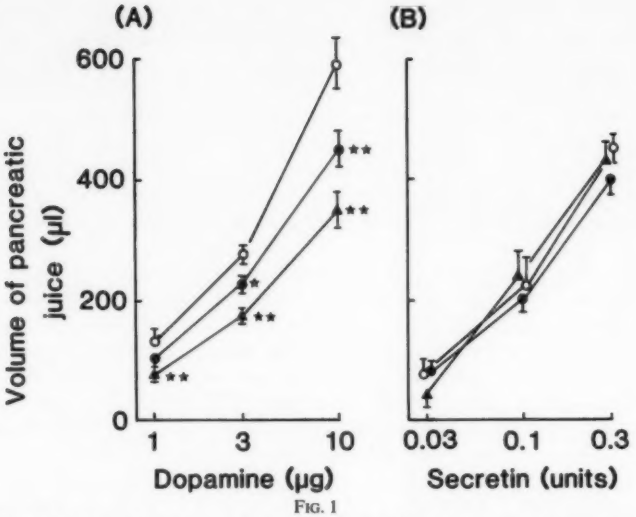

FIG. 1

Effects of verapamil on the secretion of pancreatic juice induced by (A) dopamine and (B) secretin, Control (○), during the infusion of verapamil at a rate of 50 μg/min (●) and 100 μg/min (▲). Pancreatic juice was collected from the begining until the end of the secretory response when dopamine and secretin were injected intra-arterially. Increase in pancreatic juice is expressed in volumes above the resting secretion. Each value represents mean ± S.E.M. (n = 6). Statistically different from control, $^{*}P < 0.05$, $^{**}P < 0.01$.

curve for dopamine (Fig. 1A). The reduced secretion disappeared gradually when the infusion of verapamil (100 μg/min) was stopped, and returned to the control response about 2 hr later. Intra-arterial administration of secretin (0.03, 0.1 and 0.3 units) also produced prompt increases in the secretion of pancreatic juice in a dose-related manner. During an infusion of 50 or 100 μg/min of verapamil, secretin-induced secretion was not reduced. Infusion of diltiazem (100 μg/min) or dilazep (100 μg/min) failed to alter both the dopamine- and the secretin-induced secretions (Fig. 2).

Effects of Verapamil, Diltiazem and Dilazep on the Bicarbonate and Protein Concentration in the Pancreatic Juice

The concentrations of bicarbonate in the pancreatic juice induced by dopamine (10 μg) and secretin (0.3 units) were 89 ± 4 mmol/l and 78 ± 5 mmol/l, respectively (n = 18). Verapamil, diltiazem or dilazep infused at a rate of 100 μg/min did not modify the concentration of bicarbonate in the pancreatic juice induced by both dopamine and secretin (Table I). The concentrations of protein in the pancreatic juice induced by

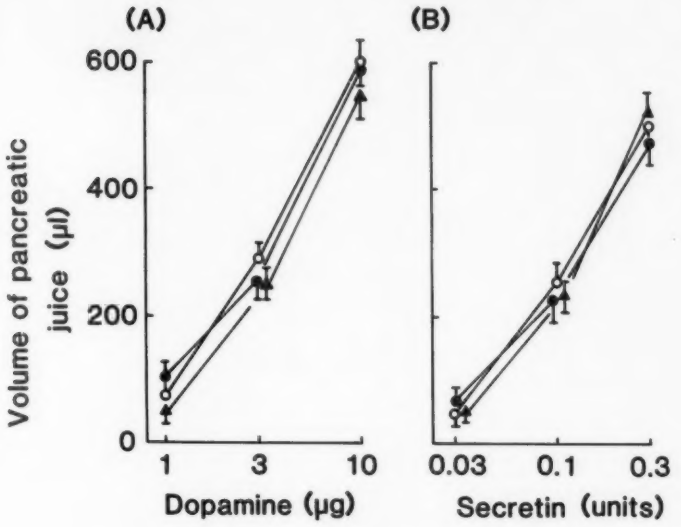

FIG. 2

Effects of diltiazem and dilazep on the secretion of pancreatic juice induced by (A) dopamine and (B) secretin, Control (○), during the infusion of diltiazem (●) and dilazep (▲) at a rate of 100 μg/min. Pancreatic juice was collected from the begining until the end of the secretory response when dopamine and secretin were injected intra-arterially. Increase in pancreatic juice is expressed in volumes above the resting secretion. Each value represents mean ± S.E.M. (n = 6).

dopamine and secretin were 24 ± 2 and 20 ± 2 mg/ml, respectively (n = 18) and they were reduced of 32 and 26 %, respectively by the infusion of verapamil (100 μg/min) (Table I). Infusion of diltiazem (100 μg/min) and dilazep (100 μg/min) also reduced the concentration of protein in the pancreatic juice induced by dopamine and secretin (Table I).

Discussion

It has been reported previously that a small dose of infused verapamil (1–5 μg/min) causes a slight and nonsignificant inhibition of the secretory response to dopamine. In contrast, this dose of verapamil caused a significant inhibition of both the response to pancreozymin and the resting secretion. These effects of the drug might be due to the action as a calcium channel antagonist (Iwatsuki *et al.*, 1982b). In the present study, we demonstrate that a large dose of verapamil (50–100 μg/min) antagonized

TABLE I

Effects of verapamil, diltiazem and dilazep on the concentration of bicarbonate and protein in pancreatic juice induced by dopamine and secretin

Treatment	Dopamine (10 μg)		Secretin (0.3 units)	
	Bicarbonate	Protein	Bicarbonate	Protein
Control	100[a)]	100[b)]	100[c)]	100[d)]
Verapamil (μg/min)				
50	94 ± 7	73 ± 8*	92 ± 13	78 ± 6*
100	93 ± 8	68 ± 9*	93 ± 8	74 ± 8*
Diltiazem (μg/min)				
100	95 ± 5	74 ± 10*	90 ± 10	79 ± 7*
Dilazep (μg/min)				
100	98 ± 8	75 ± 6*	93 ± 4	74 ± 9*

Each value represents the percent of control (n = 6).

The absolute values of control are a) 89 ± 4 mmol/l, b) 24 ± 2 mg/ml, c) 78 ± 5 mmol/l and d) 20 ± 2 mg/ml, respectively.

*Statistically different from control, $p < 0.05$.

the dopamine-induced exocrine secretion and the dose-response curve for dopamine was significantly shifted to the right. In contrast, two calcium channel blockers, structurally dissimilar from verapamil, diltiazem and dilazep, failed to alter the dopamine-induced secretion. Furthermore, the secretion induced by secretin was not modified by treatment with either verapamil, diltiazem or dilazep. Dopamine-induced secretion was also significantly inhibited by sulpiride and haloperidol (Furuta *et al.*, 1973; Honda *et al.*, 1977; Iwatsuki *et al.*, 1983), which are specific dopamine blockers in the peripheral organs (Goldberg, 1978) as well as in the central nervous system (Bartholini, 1976). Other blocking agents such as phentolamine, propranolol, atropine and cimetidine did not modify the dopamine-induced secretion (Iwatsuki *et al.*, 1983). These observations strongly indicate that the effect of verapamil on the secretion of pancreatic juice of the dog is due to its dopamine antagonist activity rather than to its calcium channel blocking action, because two other calcium blockers had no similar action. Recently, Cronin (1982) reported the dopamine antagonist activity of verapamil. A dopamine antagonist, ^{3}H-spiperone, bound to dopamine receptors in membrane preparations of the porcine anterior pituitary was displaced by verapamil *in vitro*. More direct evidence was that the inhibitory effect of dopamine on the isolated perfused ear artery was competitively antagonized by verapamil, but not by other calcium antagonists, nitrendipine and diltiazem (Johnson, 1983). As verapamil is a potent vasodilator, verapamil-induced inhibition could result

partially from the change in the perfusion flow rate. However, this possibility could be ruled out when we examined the effect of verapamil on the preparations at a constant perfusion flow rate.

In the present study, protein, but not bicarbonate, concentrations in the pancreatic juice induced by dopamine and secretin were reduced by treatment with verapamil. Similarily diltiazem and dilazep also reduced the protein concentration in the juice induced by dopamine and secretin without altering the volume of juice. Dopamine and secretin mainly act on ductular cells of the dog pancreas and stimulate the output of the pancreatic juice rich in bicarbonate and water (Furuta *et al.*, 1972; Bastie, 1977). However, it was reported that dopamine and secretin partly stimulate the acinar cells and stimulate a protein output in pancreatic juice even without significant changes in the volume of the juice (Iwatsuki and Hashimoto, 1976; Bastie, 1977). The secretion of protein from acinar cells is calcium-dependent, but secretion of electrolytes and water is not (Kanno, 1972; Iwatsuki and Hashimoto, 1976). Thus, verapamil, diltiazem and dilazep antagonize the calcium ion which is essential for the secretion of protein, and thus reduce the protein concentration in the pancreatic juice.

In conclusion, the present study suggests that verapamil reduces the dopamine-induced pancreatic fluid secretion by virtue of its dopamine antagonist activity, and reduces secretin- and dopamine-stimulated protein secretion by virtue of its calcium channel blocking action.

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Selective Local Anti-inflammatory Activity of THS-201, a New Intra-articular Steroid, in Cotton Pellet Granuloma and Monoarticular Arthritis

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Abstract—A new steroid, THS-201 was administered locally in one of a pair of inflamed sites and its local anti-inflammatory activity was compared with those of triamcinolone acetonide, methylprednisolone acetate and hydrocortisone acetate. In rats, THS-201 (80 μg/pellet) completely inhibited the formation of granulation tissue even after 21 days. In antigen-induced bilaterally arthritic rabbits, THS-201 (2 mg/joint) decreased the swelling of the treated knee joint for more than 24 days, but no such effect was seen in the opposite knee joint. The anti-inflammatory activity of the other steroids employed as reference drugs were weaker than that of THS-201. These results indicate that THS-201 is an anti-inflammatory steroids exclusively acting at the site of administration for a long period, and for this reason it might be suitable for intra-articular application in clinical use.

Introduction

Since the report of Hollander *et al.* (1951) on the effectiveness of intra-articular steroid therapy, this procedure has been widely used in the treatment of rheumatoid arthritis and other joint diseases. The treatment has been improved mainly by prolonging the action of steroids administered intra-articularly and by reducing undesirable systemic side effects. At present, microcrystalline suspensions of many different water-insoluble esters of hydrocortisone analogues are used in this procedure (Hollander, 1979). In particular, 17α,21-diacetoxy-2-bromo-6β,9α-difluoro-11β-hydroxy-1,4-pregnadiene-3,20-dione (halopredone-17,21-acetate [THS-201]) is considered to be a preferential steroid for intra-articular administration

because of its prolonged local anti-inflammatory action (Mizushima *et al.*, 1980). In a preliminary clinical trial, it has been shown that when THS-201 was injected into one of the inflamed joints of rheumatic arthritis, sustained improvement was observed in only the joint injected, but no cure was seen in the other joints (Mizushima *et al.*, 1981). The present study was carried out to confirm the anti-inflammatory effect of THS-201 in two experimental models; cotton pellet granuloma and antigen-induced monoarticular arthritis. Intensity, duration and selectivity of the local anti-inflammatory effects of THS-201 were also compared with those of some other anti-inflammatory steroids that are commonly used for intraarticular therapy.

Methods

Animals

Male rats of Wistar-strain (Tokushima Experimental Animal Laboratory, Tokushima, Japan) weighing 110–160 g and male New Zealand white rabbits (Nippon Nosan, Tochigi, Japan) weighing 3–4 kg were used. They were housed at 22° C and 65 ± 5 % of relative humidity under a controlled condition.

Drugs

THS-201 was submitted from Dainippon Ink & Chemicals, Tokyo, Japan. Triamcinolone acetonide (TA) and methylprednisolone acetate (MPA) were purchased from Hovion Soc. Ind., Lisbon, Portugal. Hydrocortisone acetate (HA) was purchased from Sigma Chemical Co. All drugs were suspended in 0.25 % sodium carboxymethyl cellulose suspension prepared with 0.9 % NaCl.

Cotton pellet granuloma in rats

According to a modified method of Winter *et al.* (1963), two cotton pellets (40 ± 1 mg) made of absorbent cotton wool were implanted bilaterally below the scapular region by a midline incision in the back of rats under light ether anesthesia. Drugs were applied to cotton pellet under sterile conditions before implantation: 0.1 ml of drug suspension was applied to a cotton pellet (treated pellet), and an equal volume of vehicle was applied to another pellet (untreated pellet). Two untreated pellets were implanted in control animals, while one treated pellet and one untreated pellet were implanted in test animals.

In a dose-response study, 8 animals of each group were sacrificed 7 days after implantation of cotton pellets, and the cotton pellets surrounded by

granulation tissue were removed, dried and weighed. The dry weight of granulation tissue was calculated by subtracting the weight of cotton pellet. The thymus was also removed and weighed.

In addition, the time course of actions of drugs tested was also examined. Forty animals of a group received implantation of one treated and one untreated pellet; 8 out of the 10 animals randomly selected from each group on day 3, 7, 14 and 21 were tested for their weights of both granulation tissue and thymus and 2 were used for histological examination. In the histological examination, the cotton pellet with surrounding tissue was fixed in formal saline and stained with haematoxylin and eosin.

Antigen-induced monoarticular arthritis in rabbits

According to a method of Consden *et al.* (1971), 10 mg of ovalbumin (Sigma Chemical, Type VII) was emulsified with 1 ml of Freund's incomplete adjuvant (Difco) containing 2 mg finely ground Mycobacterium tuberculosis H37RA (Difco), and injected intradermally into multiple sites in the dorsal neck of animals two times, with a 14 day interval. Ten days later, both knee joints were shaved and 5 mg of ovalbumin dissolved in 0.5 ml saline was injected into both knee joints through a 27 gauge needle.

The diameter of knee joints was measured every other day with a microcaliber according to the method of Blackham and Radziwonik (1977). Joint swelling was expressed as the difference in thickness (mm) measured horizontally before and after antigen challenge. Rabbits that showed more than 3.5 mm of swelling on day 14 after challenge were used for experiments. Groups of 5 animals were treated intra-articularly with 0.5 ml of drug suspension (left knee joint) and an equal volume of vehicle (right knee joint).

The rabbits were killed at the end of the experiment by an intravenous overdose of sodium pentobarbital. All joints were fixed in formal saline, decalcified, sectioned and stained with haematoxylin and eosin, and finally morphological changes in the synovial membrane were evaluated histologically using 0–3 scoring system for each of the following parameters:

1) proliferation of the lining layer; 2) lymphocyte infiltration; 3) plasma cell infiltration; 4) macrophage infiltration; 5) polymorphonuclear cell infiltration; 6) fibrin deposition; 7) neovascularization; 8) fibrosis.

The highest score would be 24 (8×3).

T

Effect of locally administered anti-inflamm
induced by c

Treatment	Dose (μg/pellet)	Dry weight of gra: Treated pellet (mg)	% Inhib.
Vehicle			37 ±
THS-201	3.2	21 ± 8**	43
	16	12 ± 6**	68
	80	9 ± 4**	76
	400	9 ± 4**	76
TA	16	17 ± 6**	54
	80	13 ± 4**	65
	400	11 ± 6**	70
MPA	80	21 ± 12**	43
	400	13 ± 8**	65
	2000	12 ± 5**	68
HA	80	23 ± 11**	38
	400	16 ± 3**	57
	2000	14 ± 8**	62

Values are means ± S.D. for 8 male rats. * and **; significant differe

TABLE I

nflammatory steroids on formation of granulation tissue
d by cotton pellets in rats

of granulation tissue				
	Untreated pellet		Body weight gain (g)	Thymus weight (mg/100 g)
b.	(mg)	% Inhib.		
37 ± 9			45 ± 8	322 ± 39
	33 ± 7		47 ± 5	295 ± 43
	35 ± 8		48 ± 10	327 ± 42
	30 ± 9		42 ± 6	315 ± 40
	31 ± 4		47 ± 4	300 ± 30
	26 ± 4**	30	48 ± 12	280 ± 57
	21 ± 12**	43	36 ± 6*	160 ± 48**
	17 ± 11**	54	21 ± 8**	38 ± 11**
	35 ± 9		43 ± 2	316 ± 40
	26 ± 8**	30	39 ± 6	185 ± 37**
	12 ± 4**	68	25 ± 5**	41 ± 12**
	31 ± 7		46 ± 6	311 ± 61
	27 ± 7*	27	47 ± 7	311 ± 48
	20 ± 4**	46	37 ± 6*	232 ± 22**

differences from vehicle-treated group at $P < 0.05$, and $P < 0.01$, respectively.

Results

1) *Cotton pellet granuloma in rats*

Local effect

The effect of THS-201, TA, MPA and HA were examined. As shown in Table I, although each of the test drugs inhibited formation of granulation tissue dose-dependently, THS-201 possessed the most potent effect, its inhibitory action being maximal at a dose of 80 μg/pellet.

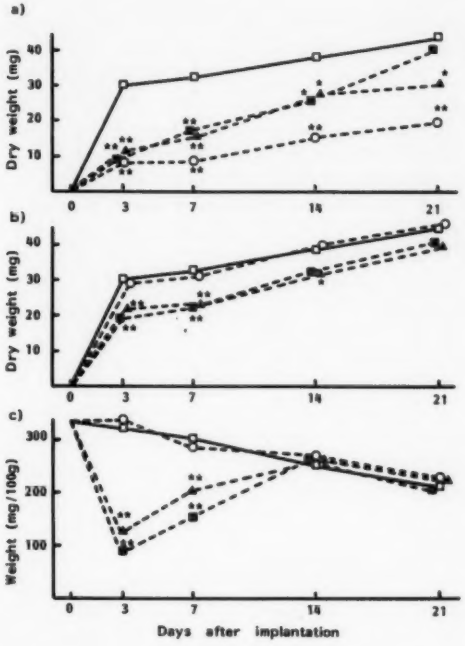

FIG. 1

Anti-inflammatory effects of locally administered steroids. a) dry weight of granulation tissue surrounding the treated pellets, b) dry weight of granulation tissue in untreated pellet and c) weight of thymus. Cotton pellets were impregnated with vehicle (—□—), 80 μg of THS-201 (--○--), 80 μg of TA (--■--) or 400 μg of MPA (--▲--). *, **; significanty differences from the control group at $P < 0.05$ and $P < 0.01$, respectively.

Systemic actions

The pellets containing TA, MPA and HA caused a dose-dependent decrease in both the formation of granulation tissue surrounding the untreated pellets and the weight of the thymus. This indicates that the treated pellets participate not only locally, but can also cause systemic effects. However, a pellet containing 400 μg of THS-201, 5 times the dose eliciting the maximal inhibition, did not exhibit such systemic actions.

Time courses of local and systemic actions

To investigate the time course of the effects of THS-201, TA and MPA, pellets containing 80 μg of THS-201 or TA and 400 μg of MPA were used, since these doses caused similar local anti-inflammatory effects in previous experiments. As shown in Fig. 1, THS-201 significantly inhibited the formation of granulation tissue even 21 days after implantation, but no systemic effect was seen throughout the experiment. TA and MPA had similar anti-inflammatory actions to THS-201 on day 3, but their local actions were slightly less on day 7 and 14, and had almost disappeared on day 21. TA and MPA showed a remarkable systemic action on day 3 and 7 that disappeared completely by day 21; the extents of systemic effects run parallel with those of local anti-inflammatory effects.

Histological examinations showed that marked cellular infiltration was observed around or into the control pellets on day 3 and formation of granulation tissue was seen in the periphery of the pellets on day 7. These inflammatory reactions were only slightly seen in the pellets treated with THS-201 but remarkable in the pellets treated with TA and MPA, obtained on day 7 and 14, respectively. Fig. 2 showes the pellets taken at 21 days after implantation. Slight cellular infiltration is seen in the pellet treated with THS-201, while marked granulation tissue is observed in all the pellets treated with vehicle, TA and MPA.

2) *Antigen-induced monoarticular arthritis*

Bilateral arthritis was elicited in rabbits by intra-articular injection of antigen into knee joints. Sequential change of joint swelling in control rabbits is shown in Fig. 3. There was no significant difference between the two knee joints, and joint swelling reached a stationary level about 14 days after challenge. The swelling of the left and right knee joints were 5.85 ± 2.18 mm and 5.89 ± 2.27 mm (mean $\pm$ S.D., $N = 36$), respectively. Animals with joint swelling of more than 3.5 mm were considered to be good responders and were used in the following experiments.

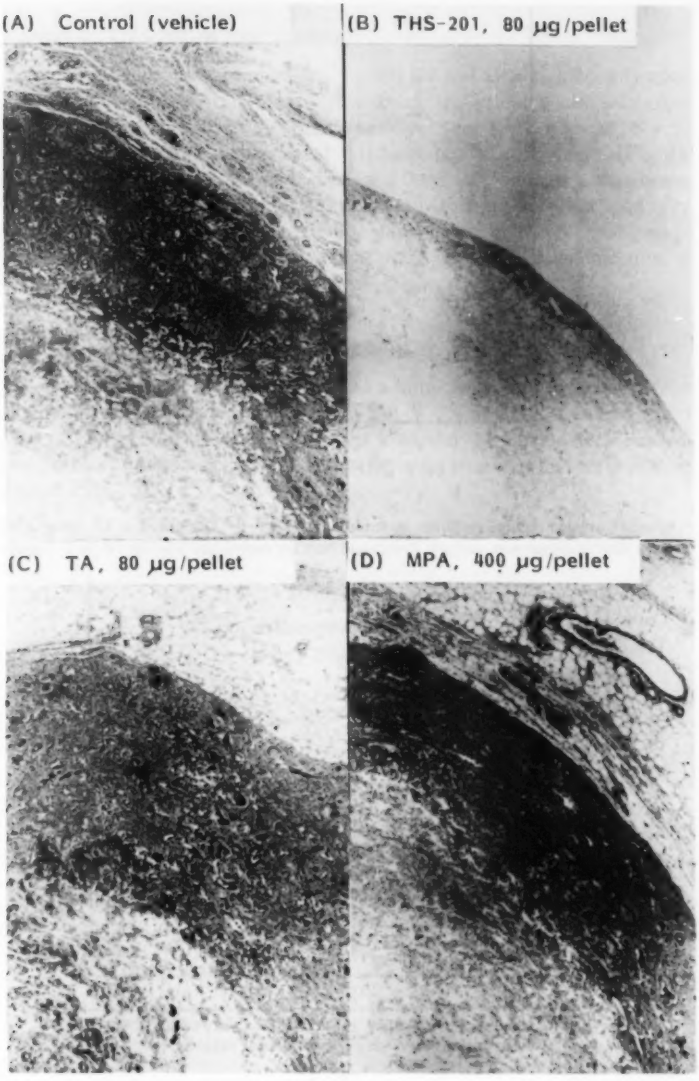

FIG. 2

Cotton pellet 21 days after implantation. Cotton pellets with (A) vehicle (control), (B) 80 μg of THS-201, (c) 80 μg of TA or (D) 400 μg of MPA. (×40).

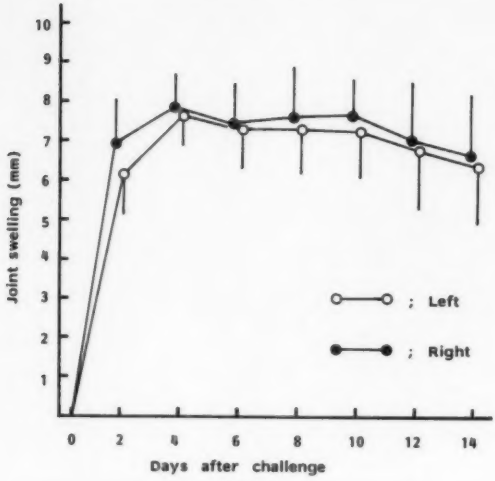

FIG. 3

Bilateral antigen-induced arthritis in rabbits. Swelling of the right and left knee joints 14 days after challenge with antigen. Points and bars are means and S.D. for values in 5 joints.

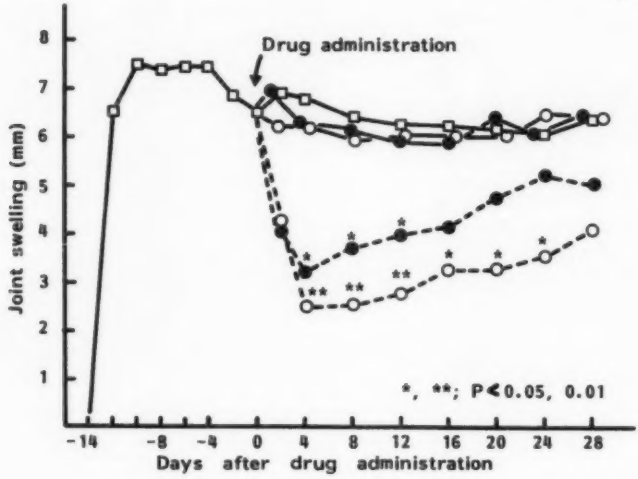

FIG. 4

Effects of THS-201 on the swelling of injected (----) and uninjected (——) joints of antigen-induced bilateral arthritic rabbits. Vehicle (□), THS-201 at 2 mg/joint (○) or THS-201 at 0.4 mg/joint (●) was administered intra-articularly 14 days after challenge.

Effect of THS-201 on joint swelling

Joint swelling of control animals (N = 10) did not alter significantly after intra-articular administration of vehicle. But intra-articular administration of THS-201 (0.4 mg and 2.0 mg/joint) into one of the swollen joints (N = 5) resulted in rapid, dose-related reduction of swelling (Fig. 4). Administration of THS-201 at a dose of 2.0 mg/joint caused a significant reduction continuing for 24 days, although subsequently its effect gradually decreased and swelling returned to the extent seen in the control group. Contralateral joints treated with vehicle were not affected by injection of THS-201.

Effects of TA and MPA on joint swelling

TA and MPA (2.0 mg/joint) were also administered intra-articularly into one of the swollen joints. As shown in Fig. 5, TA and MPA caused rapid reduction of swelling of both the treated joint and the contralateral control joint. However, the effects of TA and MPA were transient and weaker than that of THS-201.

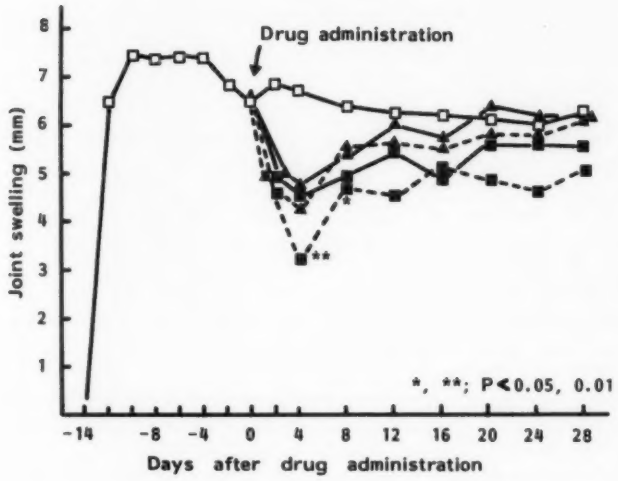

FIG. 5

Effect of TA and MPA on swelling of injected (----) or uninjected (——) joints of antigen-induced bilateral arthritic rabbits. TA at 2 mg/joint (■) or MPA at 2 mg/joint (▲) was administered intra-articularly 14 days after challenge.

Histological examination

Histological changes of the synovial membrane are summarized in Table II. The inflammation score decreased slightly on day 28 in the joint treated with 2.0 mg of THS-201, but not in those treated with 0.4 mg of THS-201, 2.0 mg of TA or 2.0 mg of MPA.

TABLE II

Effect of THS-201, TA and MPA on the synovial membrane of rabbits with antigen-induced arthritis

Group	Dose (mg/joint)	No. of joints	Inflammation scores	
			Drug-injected	Vehicle-injected
Control		10	9.3 ± 3.3	
THS-201	0.4	5	10.0 ± 4.9	9.6 ± 1.5
	2.0	5	6.8 ± 3.3	10.0 ± 1.2
TA	2.0	5	9.2 ± 3.6	9.2 ± 1.8
MPA	2.0	5	8.6 ± 0.9	9.4 ± 1.8

Value are means ± S.D. for 5 arthritic rabbits.

Discussion

Since hydrocortisone was first used in intra-articular steroid therapy in 1951, many preparations with more potent and more prolonged effects than hydrocortisone at the injection site have been used clinically (Hollander, 1979). The effects of these preparations at the site of administration can be modified by many factors, including their original anti-inflammatory actions, the rates of their elimination from the injected joints and the rates of metabolism in the joint. In this work, therefore, the local activity, systemic activity and duration of anti-inflammatory effect of various steroids including THS-201 were studied in paired sites of chronic inflammation.

A cotton pellet granuloma in rats was used as a simple experimental model to evaluate the activity of these steroids in local application. Animals were treated with two cotton pellets, one containing a test drug and other soaked with vehicle, to determine not only the local but also systemic anti-inflammatory actions simultaneously. The anti-granuloma activity observed with the treated pellet can be considered as showing local anti-inflammatory actions, and the anti-granuloma activity with the contralateral untreated pellet may reflect systemic anti-inflammatory actions. Atrophy of

the thymus and decrease in body weight gain can be considered as indications of undesirable systemic actions. Judging from results determined at 7 days after implantation of pellets, all agents tested showed potent local anti-inflammatory actions, however, a marked difference was found in systemic effects of these agents. THS-201 did not cause any change in parameters related to systemic actions, even after the administration of a dose eliciting 5 times the maximal effect on local anti-inflammatory actions. On the other hand, TA, MPA and HA showed significant systemic actions when administered at the same or lower doses than those required for their maximal anti-inflammatory actions.

Clear differences were also observed in the time courses of the effect of THS-201 and other agents. The process of inflammation induced by cotton pellets implanted subcutaneously can be classified into an exudative phase, within 72 hours, and a proliferative phase, in 3 to 6 days (Swingle and Shideman, 1972; Freeman *et al.*, 1979). In histological studies we found that infiltration of inflammatory cells into cotton pellets was extremely marked even 3 days after implantation of pellets, and that pellets were covered with granulation tissue on day 7. The cellular infiltration and the formation of granulation tissue were almost completely inhibited even 3 weeks after local administration of THS-201. Pellets containing TA and MPA showed strong local anti-inflammatory and systemic actions initially, but these actions disappeared within 14 and 21 days, respectively. These results suggest that TA and MPA were transferred into the systemic circulation rather rapidly from the site of administration.

In antigen-induced monoarticular arthritis in rabbits, reported to be a good model of rheumatoid arthritis (Dumonde and Glynn, 1962), local administration of THS-201 reduced joint swelling dose-dependently. This effect of THS-201 was observed only in the treated joint and persisted more than 24 days, although no alleviations was observed in the control joint treated with vehicle. Similar doses of TA and MPA caused slight decrease in swelling of the treated joint, but their effects were weak and short in duration. However, TA and MPA caused a definite decrease in swelling of the contralateral joint.

Histologically, no difference was found at the end of the experiment in the inflammation scores of synovitis of the injected and control joints, except for those treated with 2 mg of THS-201. These finding suggest that a recurrence may occur after elimination of the drug from the site of its administration.

From the present study, it became clear that THS-201 is extremely suitable for intra-articular application. Unlike other steroids tested, it shows only a local action and its effect is long-lasting at the site of its administration. One possible explanation for the characteristic property of THS-201 is that this compound would be absorbed very slowly from the site of administration so that the plasma concentration dose not reach a

level sufficient for systemic actions. There are reports that intra-articular injection of various steroids into patients with rheumatoid arthritis reduced the endogenous cortisol level (Bain *et al.*, 1967; Bird *et al.*, 1979) and improve the joints away from injected joint (Esselinckx *et al.*, 1978). In connection with this, it seems possible that the systemic side effects may not appear after local application of THS-201. In preliminary studies on arthritic and normal rabbits, we found that about 10 % of the initial dose of THS-201 was still present in the same joint even 28 days after its administration (unpublished data). This finding supports the conclusion that THS-201 has a prolonged effect in animals and patients.

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Studies on the Hypothermic Response of Capsaicin and its Analogue in Mice

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Abstract—Administration of capsaicin (CAP) and its related pungent, nonanoyl vanillylamide (NVA) produced significant dose-dependent hypothermic response in mice at an ambient temperature of 24° C. CAP was approximately equieffective to NVA in producing hypothermia. After large doses, desensitization occurred to the hypothermic effects of both CAP and NVA. The hypothermia produced by CAP and NVA was prevented by a small dose of thyrotropin-releasing hormone (TRH) (0.25 nmol/animal) which by itself had little effect on body temperature. Histidyl-proline diketopiperazine, a metabolite of TRH, was without effect on the hypothermic response of CAP and NVA. The result suggests that a TRH neuronal system in the brain may explain a part of the mechanism of CAP- and NVA-induced hypothermia in mice.

Introduction

Capsaicin (CAP), 8-methyl-N-vanillyl-nonenamide, is a pungent factor in red pepper. Early studies of the pharmacological actions of CAP revealed a wide profile of biological activity (Virus and Gebhart, 1979). As to the thermoregulatory reactions, parenteral administration of CAP produced a profound fall in body temperature that is accompanied by vasodilation, salivation and decreased oxygen consumption (Jancsó-Gábor *et al.*, 1970b; Szolcsányi and Jancsó-Gábor, 1973). In addition, it was reported that high doses of capsaicin or chronic treatment with the drug produced a total insensitivity to the hypothermic effects of subsequent acute administration of CAP (Jancsó-Gábor *et al.*, 1970a). A series of

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analogues of CAP have been synthesized and compared as to their potency with CAP by several biochemical and pharmacological assays (Szolcsányi and Jancsó-Gábor, 1975; 1976; Jancsó and Kiraly, 1981; Bucsics and Lembeck, 1981; Ando *et al.*, 1980). Though nonanoyl vanillylamide (NVA) has been shown to possess the activity which is equieffective to CAP, there is no analogue of CAP exceeding the potency of CAP (Szolcsányi and Jancsó-Gábor, 1975; Szolcsányi and Jancsó-Gábor, 1976; Hayes *et al.*, 1984). On the other hand, TRH is known to be abundant in the hypothalamic areas (Brownstein *et al.*, 1974) and produces hyperthermia in rabbits when injected into the hypothalamus but not into other brain areas (Carino *et al.*, 1976). It seems that TRH is one of thermoregulatory peptides possessing a physiological function in the hypothalamus. The aim of our study was to compare the thermoregulatory effect of chronically and acutely administered CAP with that of NVA. Moreover, we have assessed the ability of TRH to prevent CAP- or NVA-induced hypothermia in order to determine if this hypothermia might be mediated by brain TRH system.

Methods

Male ddY-STD mice weighing 22–27 g were used in these experiments. Mice were fed tap water and laboratory chow *ad libitum* and housed at an ambient temperature (22 + 2° C) controlled quarters with 12 hr light and 12 hr dark (21:00–9:00). Food and water were not available during 3 hr of temperature measurements. They were housed at least three days prior to their use and used only once.

Body temperature recording procedure was described elsewhere (Sakurada *et al.*, 1983). Briefly described, body temperature was recorded with as little disturbance to the mouse as possible by means of a thermister probe inserted into the rectum to a depth of approximately 2 cm. Groups of 10 mice with temperatures of 37–38° C were selected for the experiment, weighed and acclimated in the chamber for 1 hr. Each mouse was removed from the chamber, rectal temperature was recorded, and CAP or NVA was injected subcutaneously (s.c.). At 0.5, 1, 2 and 3 hr following injection, rectal temperature was redetermined. Rectal temperature recording was performed between 14:00 and 17:00.

CAP and NVA were suspended in a vehicle consisting of 10 % ethanol-10 % Tween 80-80 % saline (v:v:v) and administered in a volume of 0.1 ml/10 g. Control animals received vehicle alone. TRH and cyclo His-Pro were dissolved in sterile Ringer solution and administered intracerebroventricularly (i.c.v.) at the same time with CAP or NVA. Cyclo His-Pro was synthesized according to published procedure (Suzuki *et al.*, 1981). TRH was purchased from Peptide Institute, Inc., Osaka, Japan.

Injection schedule of short-term effect of CAP or NVA on the thermo-

regulatory response was as follows. On day 1, 4 consecutive doses of CAP or NVA (5 mg/kg, s.c.) were administered at 30 min intervals. On day 2, the effect of CAP or NVA in a dose of 5 mg/kg was examined. Moreover, on day 3, the effect of CAP or NVA (5 mg/kg, s.c.) was examined in mice following repeated administration for 2 days. Long-term effect of CAP or NVA on the thermoregulatory response was also investigated in mice treated according to the protocol described in Table I. CAP or NVA at a dose of 5 mg/kg was administered s.c. once daily for the first 4 days. Subsequently, 10 mg/kg on day 5 and 6, and 50 mg/kg on day 7, 8, 9 and 10 were administered s.c. once daily. On 12 day, 5 mg/kg of CAP or NVA was administered s.c. and body temperature was measured.

The data were analysed by a mixed two factor analysis of variance. Critical differences of the mean were calculated between treatment groups by Tukey's tests.

TABLE I

Chronic injection schedule for examining the effects of capsaicin or nonanoyl vanillylamide on body temperature

Days	Capsaicin or nonanoyl vanillylamide dose (mg/kg, s.c.)	Temperature measured
1	5	+
2	5	+
3	5	+
4	5	+
5	10	−
6	10	−
7	50	−
8	50	−
9	50	−
10	50	−
11	0	−
12	5	+

+ measured; − not measured.

Results

Effect of a single injection of CAP or NVA on rectal temperature

The time course effect of CAP and NVA was shown in Fig. 1 (Upper panel for CAP and Lower panel for NVA). Parenteral administration of either CAP or NVA to mice resulted in a dose-dependent hypothermia. A maximum dose (10 mg/kg, s.c.) of CAP or NVA produced a temperature

decrement of about 3.5–4.0° C 30 min after injection, and returned to base line within 2–3 hr. In a low dose (2 mg/kg, s.c.), NVA had a tendency to be more effective than CAP, however, in a high dose (10 mg/kg, s.c.), CAP was more effective than NVA. There were no significant changes in rectal temperature in mice injected with saline. During the falling phase of body temperature, there was a visible decrease of spontaneous motor activity.

Short-term effect of CAP or NVA on rectal temperature

Four consecutive treatments of CAP or NVA at 30 min interval produced the same degree with a single dose (5 mg/kg, s.c.) of each drug (Fig. 2; Upper panel for CAP and Lower panel for NVA). After 24 hr the mice became insensitive to the hypothermic effect at only 60 min after CAP or NVA (5 mg/kg, s.c.) was administered, though there was no change at 15 and 30 min post-injection (Fig. 2). The same phenomenon was observed after 48 hr; the efficacy with which both CAP and NVA produced hypothermia decreased with repeated administration at only 60 min post-injection.

Long-term effect of CAP or NVA on rectal temperature

The effect of previous exposure to CAP or NVA on the thermoregulatory response to subsequent exposures was examined in mice according to the protocol described in Table I. The mice which had been CAP-desensitized by repeated injections showed no hypothermic response at any time measured when 5 mg/kg of CAP was administered (Fig. 3; Upper panel for CAP and Lower panel for NVA). Administration of NVA in a dose of 5 mg/kg also produced no response when administered to mice previously desensitized with NVA. NVA is nearly as active as CAP in producing desensitization.

Effect of TRH and cyclo His-Pro on CAP- and NVA-induced hypothermia

In the first experiment, hypothermic response induced by CAP or NVA on i.c.v. administered TRH (0.25 – 1.00 nmol/mouse) was examined. TRH injected simultaneously with each drug clearly reduced the hypothermia in a dose dependent manner (Fig. 4). The hypothermia induced by each drug was significantly prevented by a small dose of TRH (0.25 nmol/mouse) which by itself had little effect on body temperature. Antagonizing effect of TRH in higher doses of 0.50 and 1.00 nmol/mouse on CAP- or NVA-induced hypothermia was more clearly observed, though TRH itself produced temperature rises of 0.5° C and 1.2° C, respectively.

In a second experiment, cyclo His-Pro, a major metabolite of TRH, was

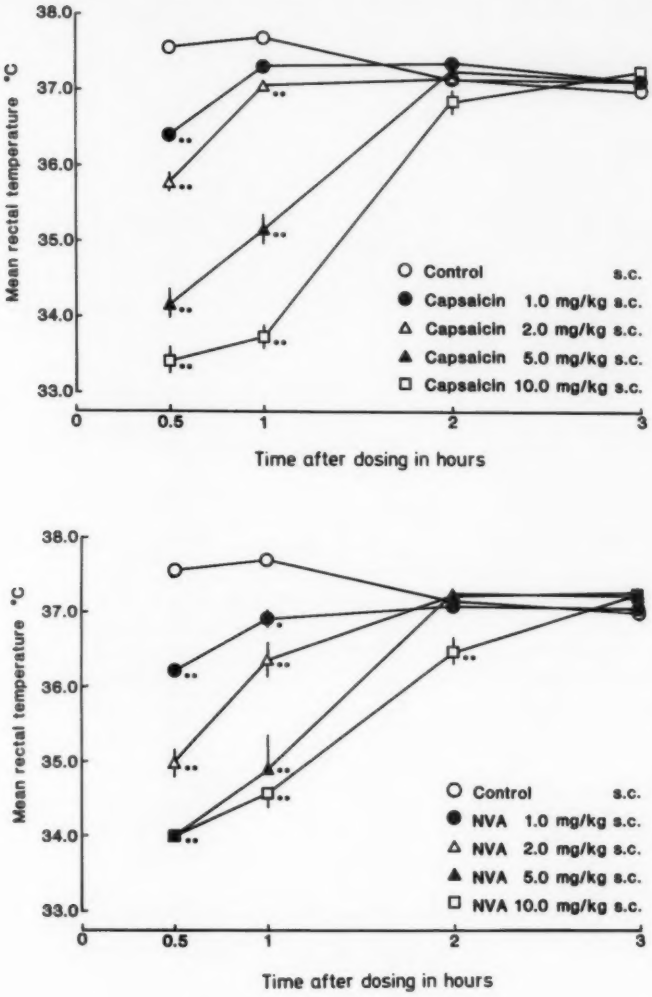

FIG. 1

Time course of hypothermia induced by different doses of capsaicin and nonanoyl vanillylamide.

Upper panel: capsaicin; *Lower panel:* nonanoyl vanillylamide. Mean values ± the standard error of the mean in 10 animals are depicted. Values which are significantly different from the saline-treated groups are indicated with * ($p < 0.05$), ** ($p < 0.01$).

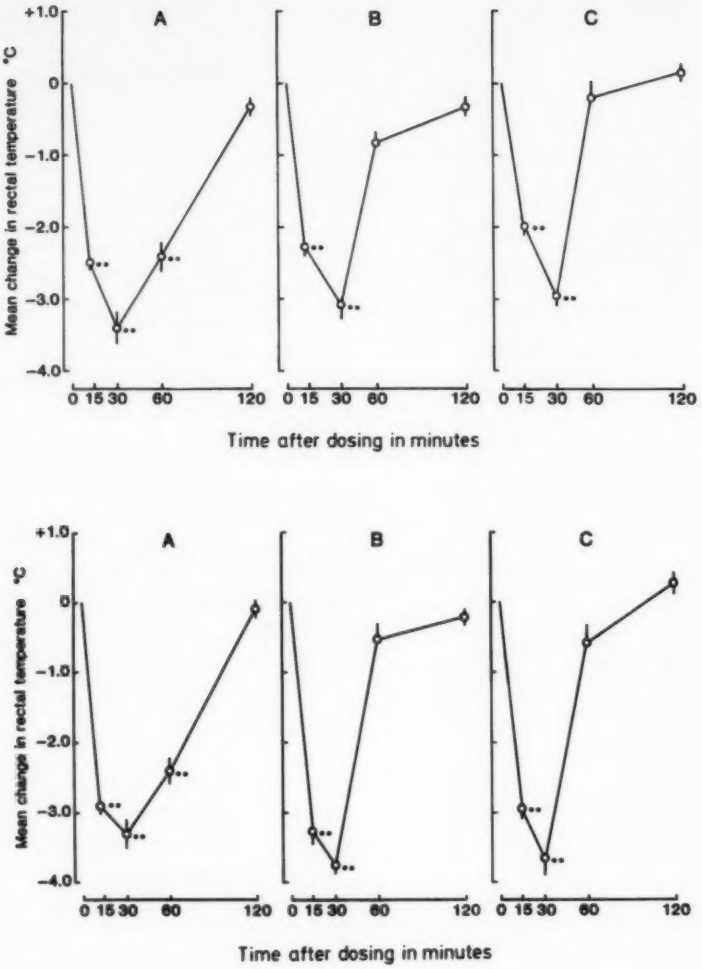

FIG. 2

Effects of repeatedly administered capsaicin and nonanoyl vanillylamide.

Upper panel: capsaicin; *Lower panel:* nonanoyl vanillylamide. A: normal mice; B: mice pretreated 24 hr before the experiment with 5 mg/kg of capsaicin or nonanoyl vanillylamide; C: mice pretreated 24 and 72 hr before the experiments with 5 mg/kg of capsaicin or nonanoyl vanillylamide. Mean values ± the standard error of the mean in 10 animals are depicted. Values which are significantly different from pre-injected level indicated with ** ($p < 0.01$).

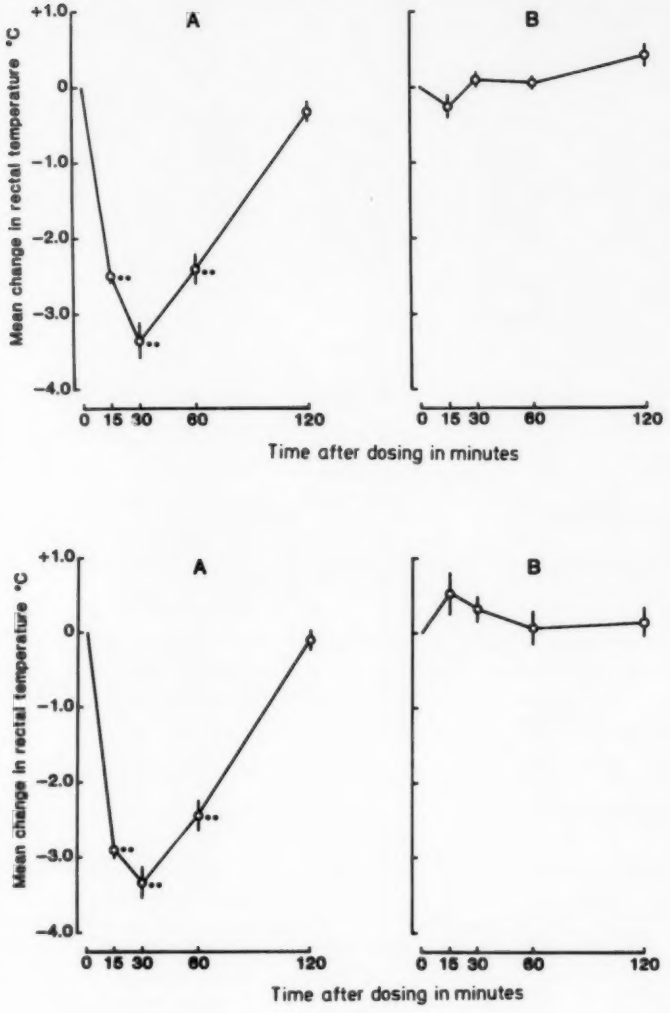

FIG. 3

Desensitizing effects of capsaicin and nonanoyl vanillylamide.

Capsaicin (*Upper panel*) or nonanoyl vanillylamide (*Lower panel*) were administered according to the protocol described in Table I. Mean values ± the standard error of the mean in 10 animals are depicted.

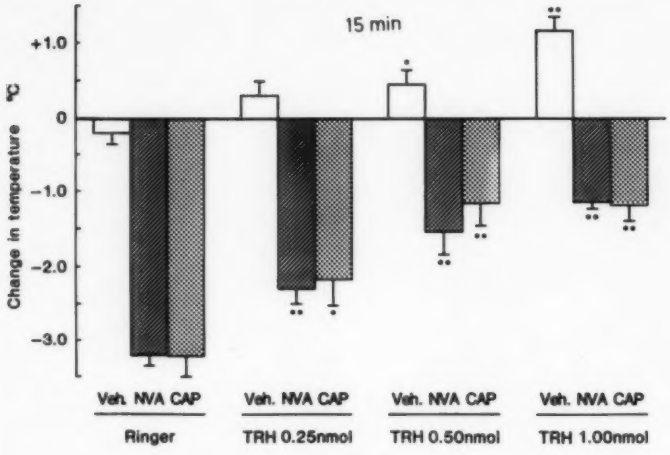

FIG. 4

Antagonism of capsaicin- and nonanoyl vanillylamide-induced hypothermia by TRH. TRH was administered i.c.v. immediately after capsaicin or nonanoyl vanillylamide. Mean values ± the standard error of the mean in 10 animals are depicted. Values which are significantly different from vehicle, capcaicin or nonanoyl vanillylamide associated with Ringer are indicated with * ($p < 0.05$), ** ($p < 0.01$).

injected i.c.v. in combination with CAP or NVA (Fig. 5). In contrast, i.c.v. injection of cyclo His-Pro (70 nmol/mouse) alone was without significant effect on rectal temperature. Cyclo His-Pro (70 nmol/mouse, i.c.v.) failed to reverse the hypothermia induced by CAP or NVA.

Discussion

NVA, an analogue of CAP, was compared with CAP with regard to the thermoregulatory effects. It has been shown that the chemical irritant CAP is capable of inducing alterations in thermoregulations in mammals. The present experiment confirms that CAP produced a profound fall in body temperature as well as the development of desensitization. NVA produced hypothermia following s.c. administration which was indistinguishable from that produced by CAP in both time of onset and duration. Further, desensitization to the hypothermic response of NVA was identical to that seen with CAP. The results of CAP and NVA on the thermoregulatory response were comparable to those reported by Szolcsányi and Jancsó-Gábor (1975); the order of effectiveness for the hypothermia-causing potency of

the compounds tested in the present study was found to be similar (CAP = NVA) to their order of effectiveness in producing desensitization. Our results are also consistent with the reports of Micevych *et al.* (1983) that intrathecally administered CAP and NVA produce a depletion of spinal SP and a profound thermal antinociception to a same extent in rats. However, the potency of NVA in producing a fall in blood pressure, in eliciting a wiping response and in releasing SP from afferent terminals in spinal cord slices was somewhat smaller than that of CAP (Bucsics and Lembeck, 1981; Skofitsch *et al.*, 1984). NVA in producing hypothermia did not exceed the potency of CAP, though NVA of synthetic CAP analogues had the highest activity in several test systems.

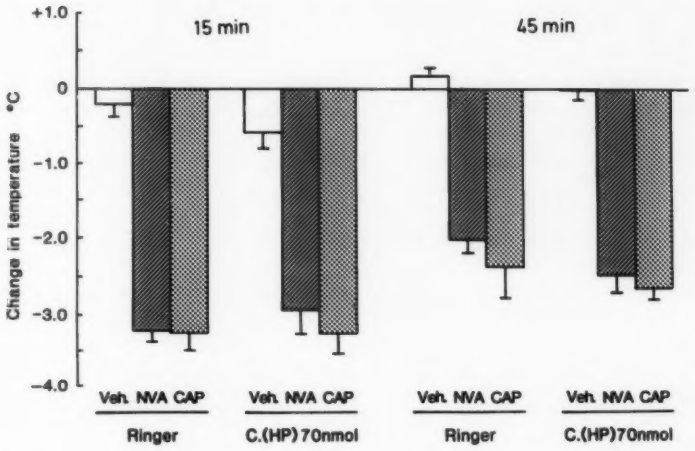

FIG. 5

Effect of capsaicin- and nonanoyl vanillylamide-induced hypothermia by cyclo His-Pro. Cyclo His-Pro was administered i.c.v. immediately after capsaicin or nonanoyl vanillylamide. Mean values ± the standard error of the mean in 10 animals are depicted.

The hypothermia produced by parenteral administration of CAP was found to cause peripheral vasodilation resulting from release of substance P from the peripheral terminals of primary afferent fibers via the axon reflex collaterals (Olgart *et al.*, 1977). On the other hand, concerning the central mechanism of hypothermic response induced by CAP, however, it is considered that CAP produced its effect on thermoregulation at sites in or near the preoptic area of the hypothalamus. The reasons were as follows: 1) The

acute injection of CAP into the preoptic area produces hypothermia similar to that produced by acute peripheral CAP treatment (Jancsó-Gábor *et al.*, 1970). 2) The chronic injection of CAP into the same area produces a complete insensitivity to the hypothermic effect of CAP (Jancsó-Gábor *et al.*, 1970). 3) Extensive lesions of the preoptic area attenuate the hypothermic effect of acutely administered CAP (Szolcsányi and Jancsó-Gábor, 1975). 4) CAP is able to pass the blood brain barrier very easily (Saria *et al.*, 1981). 5) Large doses of CAP (50–300 mg/kg) induce ultrastructural damages in the preoptic regions (Szolcsányi *et al.*, 1971). Therefore, it seems reasonable to examine the effect of TRH injected into the cerebroventricle on the hypothermic response induced by CAP in mice. It is also valuable to note TRH-effect on CAP-induced hypothermia, since TRH (pyroglutamyl-histidyl-prolineamide) is distributed in the preoptic areas as well as in the several hypothalamic nuclei of rat brain (Brownstein *et al.*, 1974). TRH induces hypothermia in the cold environment (4° C), but the same dose induces hyperthermia in the rat kept at 31° C (Parasad *et al.*, 1978). In the present experiment, a dose-dependent hyperthermic response was also seen by i.c.v. injection of TRH alone in the normal room temperature (24° C). This result is almost consistent with previous reports (Brown *et al.*, 1977; Parasad *et al.*, 1978). TRH has been shown to become cyclo His-Pro in the rat brain *in vivo* (Boler *et al.*, 1969). Studies on the relationship between TRH and cyclo His-Pro indicate that some actions of TRH are mimicked by cyclo His-Pro, whereas other actions are antagonized by this (Battaini and Peterkofsky, 1980; Bauer *et al.*, 1978; Parasad *et al.*, 1977; Parasad *et al.*, 1978). From our previous and present results, it is evident that cyclo His-Pro administered i.v.c. did not mimick TRH-induced hyperthermia (Sakurada *et al.*, 1983). I.c.v. injection with cyclo His-Pro, one of the THR metabolites, had no effect on CAP-induced hypothermia. It was, therefore, of particular interest to find that TRH injected i.c.v. clearly reversed hypothermia induced by CAP. The same results were obtained in the case of NVA. This must be considered an antagonistic action of TRH itself. Thus it is apparent that this effect of CAP or NVA was antagonized by TRH but not cyclo His-Pro, an active metabolite of TRH.

It has been suggested that there may be a connection between the hypothermic effect of CAP and 5-hydroxytryptamine (5-HT); p-chlorophenylalanine, a 5-HT depletor inhibited the hypothermic response to acute peripheral CAP treatment, whereas 5-hydroxytryptophan, a 5-HT precursor, enhanced the CAP-induced hypothermia. On the other hand, a number of examples of the coexistence of two or more putative transmitters in the central and peripheral neurones have been reported (Hökfelt *et al.*, 1980). The coexistence of TRH and 5-HT was shown in some medullary raphe neurones projecting to the spinal cord. It seems possible that CAP may act on some cells containing 5-HT together with TRH in the brain.

The results presented here provide further pharmacological evidence that there may be a connection between the hypothermic effect of CAP or NVA and the TRH neuronal system in the brain.

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